Identification of Cell Surface Assembly Mutants in *Saccharomyces cerevisiae*

by

Ann-Marie White

B.A. Biological Sciences
Hunter College of the City University of New York, 1991

Submitted to the Department of Biology in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biological Sciences

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Signature of Author .....
Department of Biology
February 2, 1999

Certified by ..
.....
Phillips W. Robbins
Professor Emeritus of Biology
Thesis Supervisor

Accepted by .....
Terry Orr-Weaver
Co-Chairman of the Biology Graduate Committee

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Abstract

The cell wall of *Saccharomyces cerevisiae* is composed of ß-linked glucans, mannoproteins, GPI-anchored glycoproteins, and chitin. Aside from protecting yeast from environmental pressures, the cell wall is one of the few structures which does not have a similar counterpart in mammalian cells. As a result, the cell wall is a good target for antifungal agents. In an attempt to identify additional genes involved in cell surface assembly, a large scale transposon mutagenesis screen was conducted. Mutants were tested for resistance or hypersensitivity to the cell wall binding drugs calcofluor white and congo red. Drug screening was performed on 15,000 mutants. Eighty-three putative positives have been isolated and the transposon disrupted gene sequenced. Putative positives were then subsequently screened with other drugs such as hygromycin B, Papulacandin, Killer Toxin, and caffeine. In addition, glucose/mannose ratios of the mutants were determined. Of the eighty-three mutants, four showed a previously unidentified "droopy bud" phenotype. These mutants were analyzed for synthetic lethality in conjunction with the primary chitin synthase, CHS3.

While some of the genes isolated have previously been shown to either directly or indirectly contribute to cell surface assembly, a considerable number have never before been associated with cell surface assembly. Also, several genes without any previously known function have been found. Interestingly, none of the chitin synthase genes and not all of the ß-glucan genes were isolated indicating that this study, though large, was far from being entirely comprehensive.

Thesis Supervisor: Phillips W. Robbins

Title: Professor Emeritus of Biology

Dedication

To all the ones who have come before, without whom, I could not stand here today.

Acknowledgments

Somewhere around my fourth year in graduate school, I began to mentally write this acknowledgement section. It started off as a joke, "Remember to thank the good folks at Burroughs-Wellcome (now Glaxo-Wellcome) for Imitrex, the wonder drug for migraine sufferers, and the only way I could finish this in one piece". Now, after spending 71/2 years here that initial tabulation comes in handy in very seriously thanking those who helped me and became a part of my life here at MIT.

First I have to thank my Mom. Coming here was my first "big trip" away from home. We spoke every night just to "check in". Even though many of our conversations were short, the constant reminder that she was there for me at any time of the day or night was a gift I can never repay. My Dad gets thanks for the car and for the car insurance. It made life much easier.

I thank Phil Robbins for his guidance, patience and support thoughout my thesis. It is an honor to be his last graduate student. I am grateful to the members of the Robbins Lab (Sue, Paul, Jane, Joan, Barbara B, Barbara O., Carlos, Debbie, Sadhana, Charlie, Humberto and Chitra) for their help, conversations, jokes, and overall good natures. At times the lab could really be called a family. Thanks to my UROPs Johanna, Amy and Craig for doing great work. I especially thank Barbara Osmond for help with this thesis and for teaching me about yeast. Without her conversations and occasional prodding I wouldn't understand yeast genetics as I do now. When I get my own lab, I'll be giving her a call to be my technician.

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was the main thing that got me through those moments when the PCR just would not work.

Rebezar and Paul have been there for me since the beginning. Harold makes the third member of my council triumvirate. I thank them for their constant quiet wisdom and for the fact that with them, I have never been alone.

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A huge thank you goes to my APO brothers for giving me an outlet for my need to serve the community and for being friends.

The SIPB allowed me to be a webmaster and ensures that my Athena account will never be doomed. This might not sound important, but having a group of course 6 majors feel that I know enough about UNIX to actually be a useful, productive, functioning member of the coolest group of computer scientists on the planet is just wonderful and is the reason why I can code.

Liz Barnes, Kenneth Weeks and Sarah Hernandez made my life here bearable. Thanks to them for eliminating most of my financial red tape.

Finally, I need to thank my Mom again. If I had one ounce of the talent that she has, I'd truly be gifted. She is, always has been and always will be, my hero. I hope that when I grow up, I can, in some small way be like her.

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I. INTRODUCTION

A. Cell Wall Overview

In addition to their intrinsic interest and importance, fungi are useful simply because they are similar to higher eukaryotes and as a result can serve as model system for the study of various cellular processes, development, morphogenesis, and cancer. Further, some species, such as *Candida albicans, Aspergillus fumigatus,* and *Cryptococcus neoformans important* because they are human pathogens. Fungal infections in mammals range from benign to lethal. With the increase in patients immuno-compromised due to AIDS, chemotherapy, transplants, and other aggressive medical treatments, there has been a substantial increase in the number of patients acquiring invasive and potentially lethal fungal infections (Sternberg, 1994). Unfortunately, there are only a few antifungal agents available. Many of these drugs attack the cellular processes of DNA replication or RNA processing. Some antifungals disrupt various mechanisms of protein processing. Still others bind to and weaken the cell membrane. However, since fungi are eukaryotes, the cellular processes and structures which the drugs attack are often similar to their mammalian counterparts. As a result, many drugs tend to have rather deleterious side effects as they destroy host cells as well as fungal cells. For example, Amphotericin B is commonly used to treat systemic fungal infections and thrush, as seen in some AIDS patients. Amphotericin B

complexes with the fungal membrane sterol ergosterol. This binding causes the membrane to lose stability and the cell subsequently dies. Unfortunately, ergosterol is similar to the mammalian membrane sterol cholesterol. Therefore, to design an effective and safe antifungal agent, it is imperative to find an essential fungal structure, which has no mammalian equivalent. The fungal cell wall is such a structure. Fortunately, the cell wall is similar throughout the fungal kingdom, so information gained using the non-pathogenic yeast *Saccharomyces cerevisiae* can be applied to other invasive, pathogenic fungi.

The cell wall is essential for the continued viability of fungi. It is the dynamic, yet rigid structure which protects the cell against environmental insults, maintains the overall shape of the cell, acts as a barrier against large macromolecules, anchors pheromone receptors, helps to maintain adequate turgor pressure inside the cell, and plays a critical role in mating and morphogenesis. Almost 30% of the total dry weight of *Saccharomyces cerevisiae* is the cell wall (Fleet, 1991). Composed of a matrix of ß-linked glucans, mannoproteins, chitin, and GPI-anchored glycoproteins, the cell wall serves as a fascinating organelle in which to study morphogenesis as well as a potential antifungal target. (Bulawa, 1991; de Nobel and Lipke, 1994; Klis, 1994; Stratford, 1994; Cid, *et.al.*, 1995; Orlean, 1996; Kollar, *et.al.*, 1997) (see Figure 1).

The cell wall is arranged in discrete layers which can be seen under low temperature and freeze substitution fixation scanning electron microscopy (Kusamichi, *et al.*, 1990; Baba *et al.*, 1987; and Cid, *et. al.*, 1995) (see Figure 2). The outer layer contains primarily mannoproteins (40%) which do not appear to play a dominant role in maintaining the structural stability of the cell; however, there is evidence that they are important in cell adhesion and morphogenesis (Zlotnik *et al.*, 1984, Koch *et al.*, 1980, Orlean 1996, Lipke and Kurjan 1992, Kollar *et. al.*, 1997, and Stratford *et. al.* 1992). Hot citrate extraction is commonly used to study the carbohydrate side chains of these cell wall proteins. (Ballou, 1990). While this method destroys some noncovalent interactions, it has been used to determine that mannoproteins consist of a core of 8-15 N-glycosidically linked α-(1,6)-mannose residues linked to asparagine with a branched outer chain consisting of up to 200 α-(1,2) and α-(1,3)-mannose residues. In addition, there are smaller O-sidechains linked to serine/threonine. These 0-linked chains contain anywhere from one to five α-(1,2)-linked mannose residues (see Figure 3).

The inner cell wall layers provide mechanical strength and contain ß-linked glucans with interspersed chitin chains (Zlotnik *et al.*, 1984). The β-glucans make up 30-60% of the cell wall and can be separated into classes based on acid and alkali insolubility, and β-(1,3) and β-(1,6)-

glucanase digestion. Extraction with hot alkali produces a fraction containing chitin, β-(1,3) and β-(1,6)-glucan. Acid extraction produces a fraction without chitin but of roughly equal glucan composition suggesting that chitin crosslinking renders glucan alkali insoluble. β-(1,3)-Glucan has a branched structure containing about 1500 residues, compared to β-(1,6)-glucan which contains an average of 140 residues. (Klis *et. al.*, 1997 and Klis 1994). Recent studies show that β-(1,6)-glucans link mannoproteins, chitin, and β-(1,3)- glucans into a structural lattice that forms a flexible yet strong building block for the cell wall (Kollar *et.al.* 1997) (see Figure 4).

Chitin is a homopolymer of GlcNAc which is produced by the reaction nUDPGlcNAc $\rightarrow$ [GlcNAc-β-1,4-GlcNAc]$_{n/2}$+nUDP. Though chitin comprises only 1-2% of the cell wall in *Saccharomyces cerevisiae*, it has a high tensile strength and is essential for the integrity of the cell wall. Though it is found in small amounts delocalized throughout the cell wall, it is primarily made at the end of G_1 and deposited in the cell wall at the location where the daughter cell begins to bud. Chitin also forms the primary septum, which initially separates the mother and daughter cell and remains as the bud scar in the mother cell after mother and daughter have separated.

Chitin synthesis probably occurs in the plasma membrane. Nascent strands of chitin are extruded across the plasma membrane and hydrogen bond to form chitin in the cell wall. During sporulation, nascent strands of

chitin are deacetylated to form chitosan in a process, which is not fully understood. In *Saccharomyces cerevisiae* chitin can be detected in 3 ways. Dyes such as Calcofluor White and Congo Red selectively bind chitin and other fibrous polysaccharides. Wheat germ agglutinin binds GlcNAc and has a high affinity for β-(1,4)- linked GlcNAc polymers. Finally, there are colorimetric assays which can quantitate chitin in the cell (see Figure 5) (see Table 1).

Several cell wall proteins have a C-terminus modification which allows for the attachment of glycosyl-phosphatidyl-inositol (GPI). This hydrophobic terminus is cleaved off in the endoplasmic reticulum and replaced by the highly conserved GPI anchor (ethanolamine-P-6, Man-α1,2-Man-α1,6-Man-α1,4-GlcN-α1,6-inositol) (see Figure 6).

B. Specific Genes Involved in Cell Surface Assembly

The complete *Saccharomyces cerevisiae* sequence is catalogued in several databases. The Saccharomyces Genome Database (SGD, http://genome-www.stanford.edu/Saccharomyces), MIPS: The Yeast Genome Project (MIPS, http://speedy.mips.biochem.mpg.de/mips/yeast/), Yeast Proteome Database(YPD,http://quest7.proteome.com/YPDhome.html), and GENBANK (http://www.ncbi.nlm.nih.gov), the most popular databases for yeast, each have internal and cross-database cross referencing capabilities. In addition, yeast DNA can be compared with sequence from

other organisms to identify potential homology in sequence and function. This valuable resource allows for easier identification and analysis of genes unique to yeast that are involved in cell wall assembly. Of the 6,000 genes in the genome, it is thought that at least 600 and up to 1200 are involved in the construction and maintenance of the cell surface architecture.

1. Chitin Synthesis

Classical genetic analysis has shown that there are three chitin synthases in *Saccharomyces cerevisiae* which are used to build chitin chains under specific conditions and at specific times (Bulawa, 1993). All three chitin synthases catalyze the identical reaction. However, they differ in where they localize chitin (see Figure 7). Chitin synthase I *(CHS1)* is required for normal budding under acidic conditions (Bulawa, 1993). However, Δ*chs1* mutants show no obvious phenotypic differences with respect to chitin localization, mating or budding, but, they lack much *in vitro* chitin synthase activity. *CHS1* is 10 to 20 times more active in extracts than *CHS2* or *CHS3.* It is thought that *CHS1* deposits "repair" chitin in the neck between the mother and the daughter cell. Chitin synthase II *(CHS2)* is required to maintain proper cell morphology and normal cell separation, including septum formation (Sliverman *et.al.*, 1988; Bulawa, 1993). Chitin synthase III *(CHS3)* produces greater than 90% of all the chitin in the cell and its expression is governed by *CHS3, CSD4,* and

CAL3 (Bulawa, 1993 Bulawa, *et.al.*, 1986). It is responsible for the synthesis of chitin in the ring and the lateral wall. *CHS3* also regulates the synthesis of nascent strands of chitin which form chitosan, and it is the chitin produced by *CHS3* which is linked to β-(1,3)-glucan. While Δ*chs1*/Δ*chs2* and Δ*chs1*/Δ*chs3* are viable Δ*chs1*/Δ*chs2*/Δ*chs3* is not, showing that chitin synthesis is essential. (Bulawa and Osmond, 1990, Shaw *et.al.*, 1991, Bulawa, 1992, Bulawa, 1991) (see Table 2).

2. β-Glucan Synthesis

Extensive analysis of Killer Toxin resistant mutants *(KRE)* by Howard Bussey and others have allowed the isolation of many of the genes involved in the ß(1,6)-glucan synthesis pathway. Killer Toxin binds to chains of ß-(1,6)-glucan. As a result, cells with decreased levels of ß-(1,6)-glucan are resistant to Killer Toxin. At least six *KRE* genes *(KRE1, KRE2/MNT1, KRE5, KRE6, KRE9, KRE11)* seem to be directly involved in ß-(1,6)-glucan synthesis (Klis, 1994; Brown and Bussey, 1993) *KRE2/MNT1* is involved in the elongation of O- and N-linked carbohydrate chains (Meaden *et.al.*, 1990). *KRE5* is epistatic to all *KRE* mutants (Meaden *et.al.*, 1990). *KRE1* mutants produce only 40% of the normal amounts of β-(1,6)-glucan (Brown *et.al., 1993,* Boone *et.al.*, 1990). *KRE11* mutants have a 50% reduction in β-(1,6)-glucan and are synthetically lethal in combination with Δ*kre6* and seem to have UDPGlc binding problem. *KRE9* is

synthetically lethal in combination with Δ*kre6*, Δ*kre11* and has an 80% reduction in β-(1,6)-glucan. *KRE6* mutants have a 50% reduction in β-(1,6)-glucan and are synthetically lethal in combination with a deletion in protein kinase C 1 (Δ*pkc1*). It is clear that the *KRE* genes act at different stages of the secretory pathway. As a result, it is thought that the synthesis of β-(1,6)-glucan begins in the secretory pathway, in contrast to the synthesis of β-(1,3)-glucan.

β-(1,3)-Glucan is synthesized in the plasma membrane by *FKS1p*, *FKS2p* and *RHO1p*. *FSK1* is required for bud expansion and is expressed primarily during G_1/early S phase in the tips of emerging buds. *FKS2p* is regulated by mating pheromone. *RHO1p* is co-localized with *FKS1* and is also involved in the regulation of actin at the bud tip. It is also involved in general cell wall synthesis regulation (Mazur *et.al.*, 1995, Cabib, 1980). In addition to the genes already mentioned, *KNR4, GNS1, HKR1, GAS1* and *GGP1* are involved in the regulation of β-(1,3) and β-(1,6)-glucan synthesis (El-Sherbeini and Clemas, 1995; Fishel *et.al.*, 1993; Hong *et.al.*, 1994; Orlean, 1991; Yabe *et.al.*, 1996) .

C. Summary Of Thesis Work

In an effort to identify additional genes involved in cell wall synthesis, two separate types of screens were conducted. First, a transposon mutagenized yeast genomic library was screened for resistance

or hyper-sensitivity to the cell wall binding drugs calcofluor white (CFW) and congo red (CGR). Second, a synthetic lethal screen was established to identify genes which interact with CHS3.

The two classic methods used for generating novel mutations in large numbers of yeast are ultraviolet (UV) irradiation and ethyl methanesulfonate (EMS) mutagenesis. UV radiation produces photodimers in DNA which usually result in transition mutations. In addition, UV light can create deletions, duplications, transversions and frame-shifts. EMS mutagenesis primarily induces point mutations. The main advantage for using either EMS or UV is the speed with which mutagenesis can occur and the large number of cells which can be mutagenized at any given time. The problem with these mutagens is that it is not trivial to determine which gene has been mutated, whether or not there is more than one mutation in the cell, and what type of mutation has occurred. It is for this reason we chose transposon mutagenesis. Transposon mutagenesis allows random mutations to be created that have the advantage of being "tagged" with the transposon and a plasmid. As a result, the genes can be easily isolated. In this particular instance, *LacZ* :AMP:*Leu2*:Tn3 insertions were introduced into a yeast genomic library in *E. coli.* While it has been shown that there are "hotspots" for transposon insertion in this library, it is also clear that the majority of genes in the library have insertions. When presented with a linear piece homologous sequence, yeast will

preferentially undergo homologous recombination. Thus, the transposon mutagenesis scheme tends to generate single, non-tandem insertions in yeast genes of interest. After mutagenesis and screening, it is easy to recover the mutated gene by transforming with a linear recovery plasmid containing AMP and an *E. coli* origin of replication. After DNA isolation, digestion, ligation and transformation, the yeast fragment can be isolated from *E. coli* and sequenced using plasmid primers. Since the entire yeast genome has been sequenced, it is easy to use the databases to find the location of the mutant gene.

1. Transposon Mutagenesis

For the transposon mutagenesis study, the mutagenized genomic library was kindly provided by Dr. Michael Snyder. Two yeast strains were chosen for study, PRY441 and AWM3CΔ630. There were several factors involved in strain selection. We realized that since we were introducing homologous sequence into the strains and relying on recombination events to cause our mutations, we wanted to have as little extraneous sequence available as possible. As a result we chose strains without the yeast 2μm plasmid. In a preliminary screen with a 2μm containing strain, a substantive number of transformants contained an integration in the 2μm DNA.

The background sensitivity of the strains to CFW and CGR was also a concern. The background resistance of some strains of yeast to CFW and CGR can be relatively high. We wanted a strain with a lower native resistance to the dyes so that resistance would be easier to score. However, we did not want it to be so low that sensitivity would be difficult to detect. The two strains have different, but low resistances. Finally, we decided to mutate haploid strains rather than diploid. The rationale for this was that, while we would miss lethals, we would not have an additional step of tetrad analysis to detect the initial mutations.

Mutagenesis of the haploid yeast strains PRY441 and AWM3CΔ630 was carried out according the protocol outlined in Burns *et al.*, 1994. The mutagenized genomic library was digested with *Not*I to release the yeast DNA with transposon insertion from plasmid pHSS6-Sal (see Figure 8). Haploid strains PRY441 and AWM3CΔ630 were transformed with the yeast DNA fragments. Transformants were selected on synthetic complete medium lacking leucine. Transformants were picked and placed in 96 well dishes containing liquid rich, complex media with glucose as a carbon source (YPD). Each dish also contained the parent strain, a previously isolated resistant mutant and a previously isolated hyper-sensitive mutant. After overnight incubation at 30°C, transformants were replica plated, using a pronged manifold, onto solid YPD and solid rich complex

media with glycerol as a nonfermentable carbon source (YPG) (see Figure 9). All remaining cells were frozen, for permanent storage, in 96 well plates. In an effort to remove one class of respiratory mutants (petites), transformants that failed to grow on YPG plates were excluded from further consideration. Petites grow slowly, and could be confused with hyper-sensitive mutants.

Yeast strain PRY441 is very sensitive to calcofluor white, with growth arrest occurring at concentrations of 5μg/ml. Therefore, to prevent spurious data due to colony saturation and titration of calcofluor white and congo red, dilutions of the transformants were performed. Serial dilutions were carried out by replica plating, from the YPD plate into 96 well dishes containing 100μl/well distilled H_2O. While this does call to question the possibility of retrieval of osmotically sensitive mutants, control experiments showed that this was not a critical issue. AWM3CΔ630 is not as sensitive to CFW and the multiple dilutions were not done with this strain. Approximately 15,000 transformants were screened, at various dilutions, on YPD containing varying concentrations of CFW or CGR. Calcofluor white hydrogen bonds to nascent chitin chains and prevents the formation of microfibrils of chitin (Elorza *et al.*, 1983). It also seems to block the formation of hydrogen bonds involving other macromolecules such as mannoproteins (Murgui *et al.*, 1985). As a result, large aggregates

are formed when mother and daughter cell fail to separate properly. Congo red acts in a similar fashion to CFW, except that it blocks the assembly of ß-(1,3)-glucan microfibrils (Kopecka and Gabriel, 1992). Mutants with decreased amounts of glucan or chitin in the cell are, in general, CGR or CFW resistant. Therefore, by screening for resistance or hyper-sensitivity to CFW or CGR, we hoped to be able to identify genes involved in the synthesis of the cell wall (Ram *et al.*, 1994). While we did not expect to recover single essential genes, the redundancy of function of the genes in the yeast genome made it seem possible that we might find additional genes important for the assembly of the cell wall.

This screen has also allowed the isolation of genes whose absence effects cell wall assembly indirectly. Careful analysis of additional phenotypes was necessary to identify genes whose action only peripherally effects the cell wall. Of approximately 15,000 mutants screened, several hundred were either resistant or hyper-sensitive to CFW and CGR. Dilutions of approximately 5000, 500, 50 and 5 cells were spotted on varying concentrations of CFW and CGR. The genes disrupted by the transposon were sequenced using a forward *LacZ* primer and the resulting sequences were analyzed through NCBI BLAST to search for sequence homologies (see Figure 10). In order to determine whether or not the phenotypes recovered were the result of the transposon integration alone, southern analysis and/or backcrossing of the mutants was carried

out. In the end, close to 200 mutants were determined to have a drug phenotype due solely to the transposon integration, 82 of which are described in Lussier *et al.,* 1997. Of the 82, 50 genes were previously identified, but most had not been previously connected to the cell wall. Seventeen genes encoded proteins which could have direct relevance to cell wall biosynthesis. Fifteen genes had no previously identified phenotype(Lussier *et. al.,* 1997).

After identifying the 82 cell wall mutants, we proceeded to further characterize them. To assess the chitin and glucan levels in the cells, we measured the sugar composition (glucan/mannose ratios) of stationary-phase cells (Ram *et.al.,*1994). In addition, we conducted several drug tests: (K1 Killer Toxin resistance, Echinocandin sensitivity, Hygromycin B sensitivity, Papulacandin B sensitivity, Nikkomycin Z sensitivity, and caffeine sensitivity) to establish a more complete phenotype.

K1 Killer Toxin binds to the ß-(1,6)-glucan receptor on the cell surface and cells with a reduced amount of ß-(1,6)-glucan are resistant. The echinocandins are fungicidal lipopeptides which noncompetitively inhibit the synthesis of ß-(1,3)-glucans (Sawistowska-Schroder *et al.,* 1984). They are particularly effective against *Candida albicans.* (Debono and Gordee, 1994), Hygromycin B is an aminoglycoside which does not affect "normal" yeast cells. However, cells with N-glycosylation defects are sensitive to it (Ballou *et.al.,* 1990). Papulacandin B is a glycolipid which

non-competitively inhibits ß-(1,3)-glucan synthase (Debono and Gordee, 1994). Its action is similar to that of the echinocandians. All the papulacandins have a core cyclic moiety with antifungal activity being conferred by the side chains. Nikkomycin Z is a nucleoside di-peptide which is a competitive inhibitor of chitin synthase. Interestingly, it seems to inhibit CHS1 and CHS3 with more efficiency than CHS2 (Georgopapadakou and Tkacz, 1994). Paper disk tests using Killer Toxin, Echinocandin, hygromycin B, Papulacandin B, and Nikkomycin Z were conducted. Testing this way, rather than by streaking, had the advantage of being easily done on a large scale and, by measuring zones of inhibition around the disks loaded with the drug, were semi-quantitative.

Overall stability of the cell wall was tested by determining temperature sensitivity, osmotic sensitivity, zymolyase sensitivity, and sensitivity to caffeine. Caffeine seems to exacerbate the weakened cell wall found in cells with mutations in the PKC-MPK1 signal transduction pathway (Costigan *et al.,* 1992, Posas *et al.,* 1993). Zymolyase in a hypotonic solution will lyse weakend cell walls.

After all the mutants were further characterized in the above mentioned fashion, we examined morphology in an attempt to select mutants for further study. We found 4 mutants (ECM2, ECM5, ECM19, and ECM20) which exhibited an abnormal, "droopy bud" phenotype. Upon closer inspection, we found that they have extremely abnormal CFW

staining. The mutations do not seem to effect osmotic stability or temperature sensitivity of the cells. They all have an increased mannose/glucose ratio, an increase in GlcNAc, and are hygromycin hypersensitive. The four gene sequences are not similar to one another. In addition, the mutations do not seem to confer glycosylation defects.

2. Synthetic Lethal Screen

In another study to identify cell wall mutants, we conducted a synthetic lethal screen. In this screen, the object was to identify mutations which are lethal only in combination with other specific mutations. We used the *ade2 ade3* red/white color assay, developed by Koshland, to determine which mutants are synthetically lethal with Δ*chs3*. The obvious advantage is the specificity with which one can identify genes that interact directly or indirectly with specific genes or gene families (*Microbiological Reviews* 59:94-123, 1995), in this case CHS3, the primary chitin synthase gene. However, since we used UV to mutagenize the initial strain, the subsequent cloning and identification of the gene was not as simple as with the transposon mutagenesis study.

The screen was conducted as follows: The initial strain, PRY487, carried *ade2, ade3,* and *chs3* disrupted by HIS3. In addition, it carried a CEN plasmid with CHS3 and ADE3. The *ade2* causes the strain to accumulate a red pigment. However, since *ade3* is epistatic to *ade2,* a

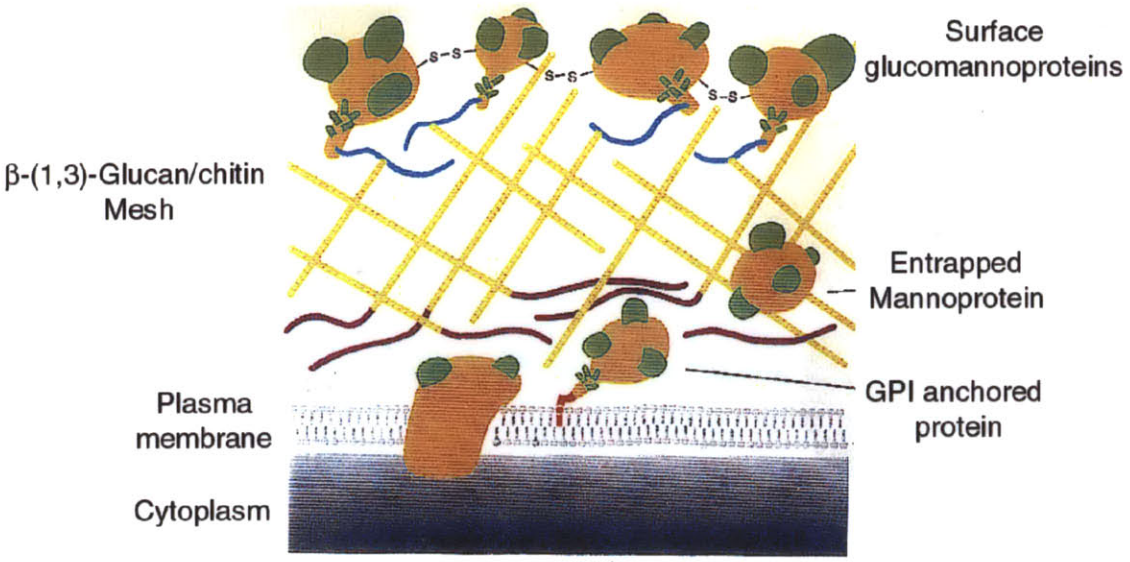
strain with both mutations is white. Thus, it is easy to tell if a particular colony can lose the plasmid, since after loss a colony will begin to lose the red pigment and will "sector". After UV mutagenesis, the cells are plated and screened for non-sectoring colonies. Of $1x10^5$ cells, 23 mutants failed to sector. However, to insure that the non-sectoring phenotype was indeed due to the mutation and not due to an integration event which would place the ADE3 from the plasmid onto the chromosome, the mutants were backcrossed to PRY398 (the strain used to construct PRY487). The diploids were screened for their sectoring ability. All 23 mutants showed a recessive sectoring pattern; however, only 10 of the 23 sectored when transformed with a tester plasmid which carried CHS3 but not ADE3. Of those 10 only 5 were healthy enough to continue successful characterization. These mutants were subjected to drug tests as described above. Three complementation groups were identified as being essential with CHS3: FSK1, SRV2, and ANP1 (Osmond *et.al.*, unpublished data). In addition, at least one of the "droopy bud" mutants was shown to be synthetically lethal in combination with *Δchs3*.

In summary, this thesis details one large scale attempt to identify genes involved in cell surface assembly. In addition, it gives an overview of the current genetics and molecular biology of cell surface synthesis and architecture in *Saccharomyces cerevisiae*.

D. Figures

Figure 1

Cell Wall of *Saccharomyces cerevisiae*

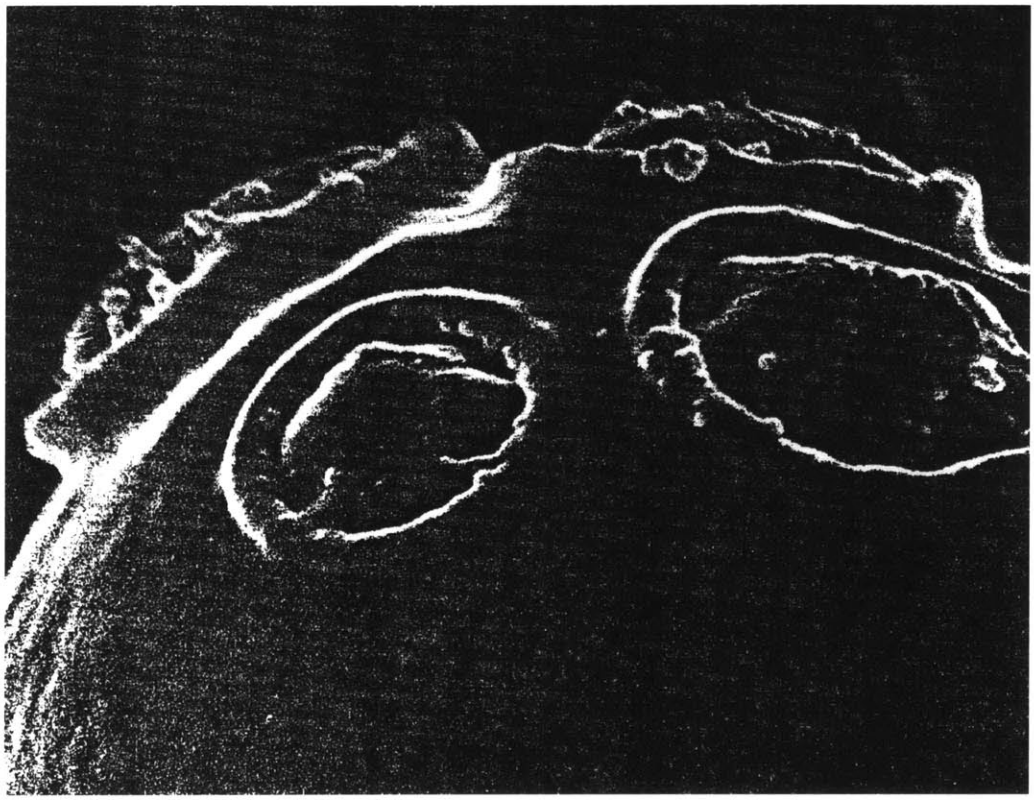


The cell wall of *Saccharomyces cerevisiae* contains Glucans, Mannoproteins, GPI-anchored proteins, and Chitin*

*Georgopapadakou and Tkacz, 1995

Figure 2

EM Photo of Bud Scars*

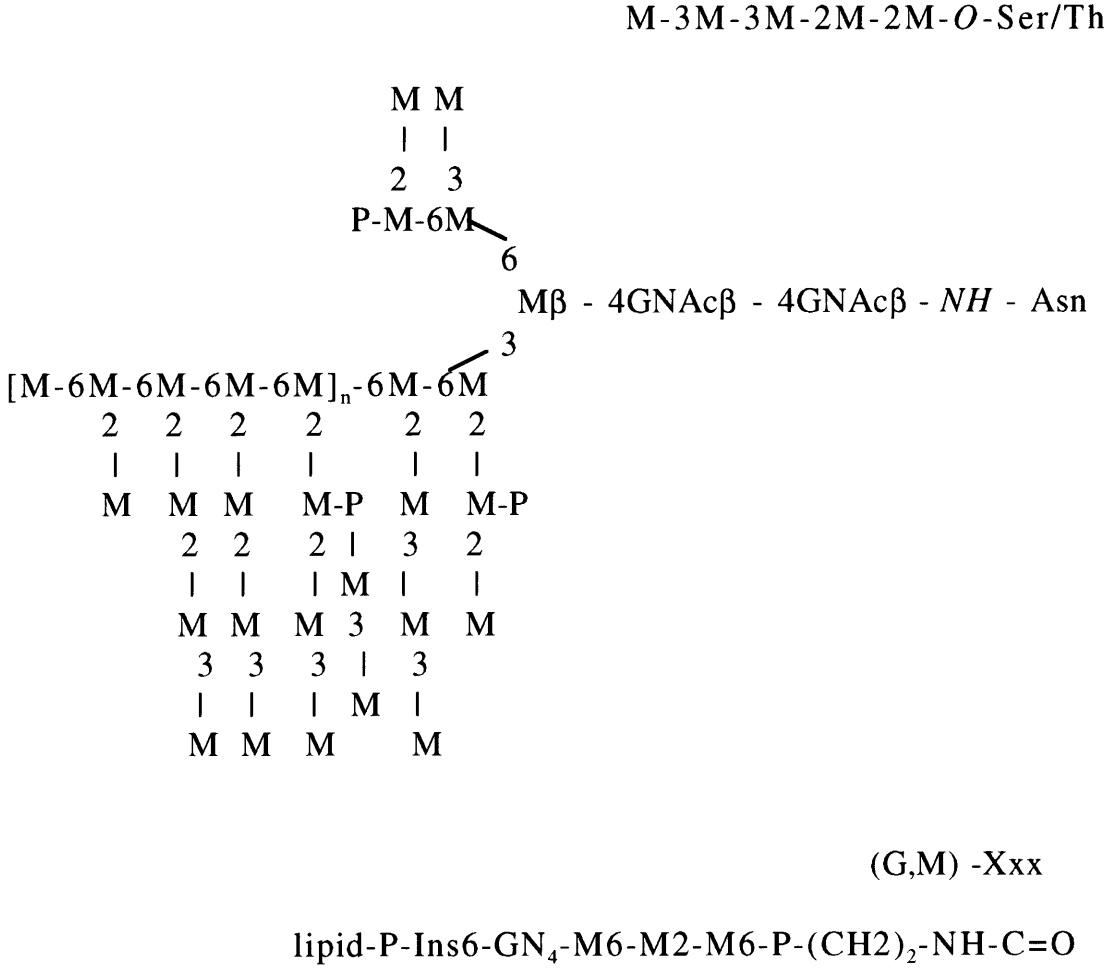


Chitin is localized in the bud scar and mother-bud junction.

*This photo taken from Stratford (1994).

Figure 3

Mannoprotein Structures

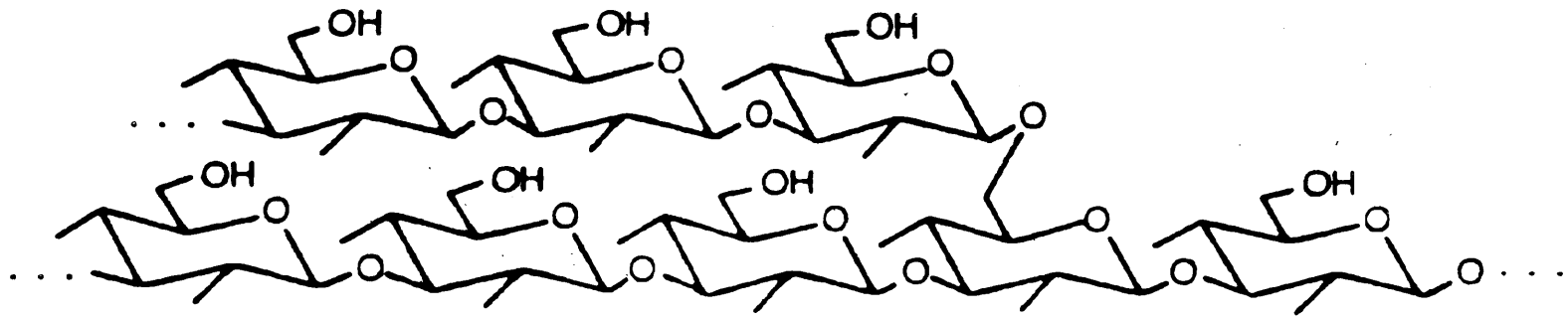


Serine/threonine *O*-side chains contain 1-5 mannose residues. Asparagine *N*-side chains vary in the number of repeating units up to 200. Gluco-manno chains consist mainly of β-1,6 linked glucose residues and α-1,6 mannose residues (Klis, 1994).

M=mannose
G=Glucose
GN=glucosamine
GlcNAc =N-acetyl-glucosamine

Figure 4

Glucan Structure

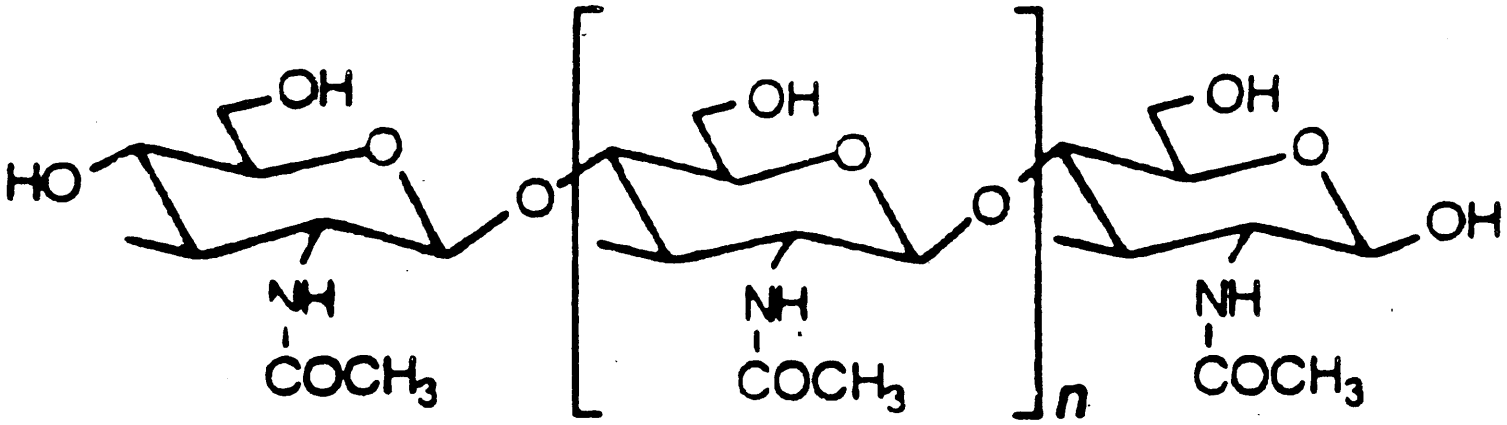


β-1,3-Glucan is a branched structure containing up to 1500 residues.

β-1,6-Glucan contains an average of 140 residues.

Figure 5

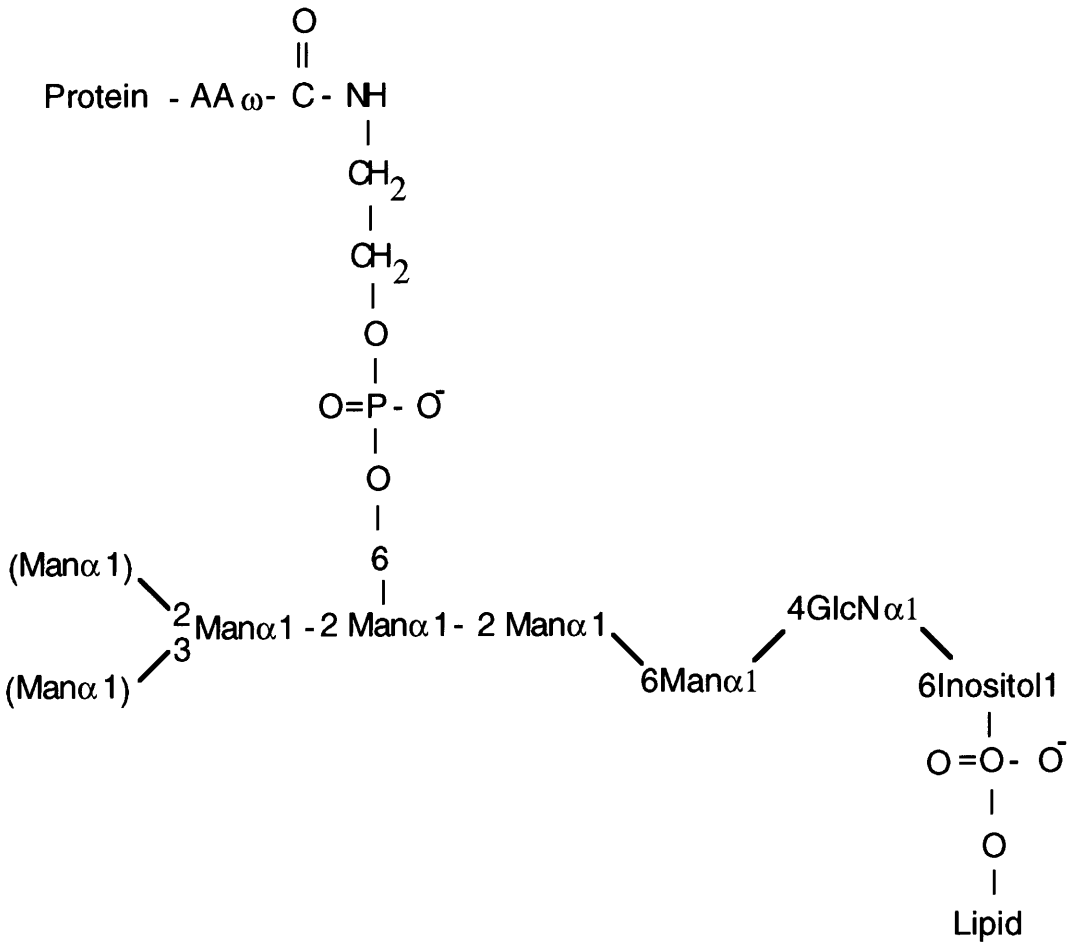
Chitin Structure



Chitin is a homopolymer of GlcNAc which is produced by the reaction nUDPGlcNAc [GlcNAc-β-1,4-GlcNAc]$_{n/2}$ +nUDP.

Figure 6

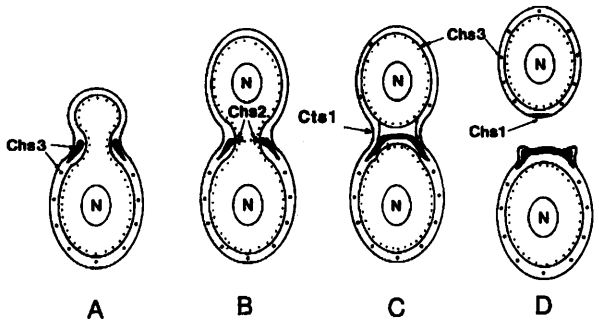
GPI-anchor Structure



This gpi modification occurs in the ER, where the C-terminal GPI-anchor-addition signal is replaced by this preassembled GPI anchor. In the Golgi another mannose residue is added either at the 2- or 3- position of the fourth mannose residue. (Klis, 1994)

Figure 7

Chitin Localization*



CHS1 deposits "repair" chitin in the neck between the mother and the daughter cell.
CHS2 deposits chitin at the septum.
CHS3 deposits chitin in the lateral cell wall and at the mother daughter junction.

*This figure designed by C.A. Specht.

Figure 8

Transposon Mutagenesis

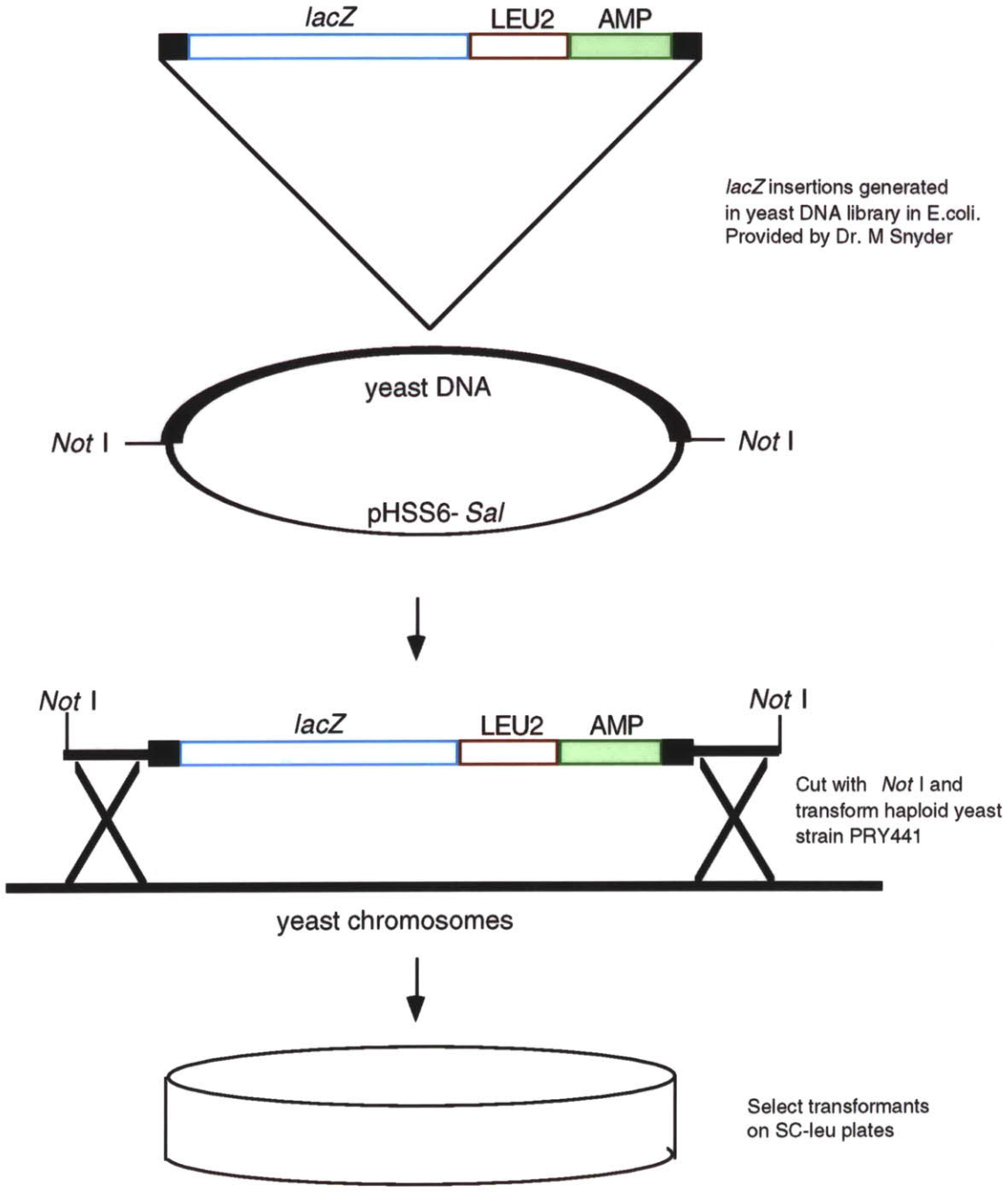


Figure adapted from Burns *et al*

Figure 9

Calcofluor White/Congo Red Screen

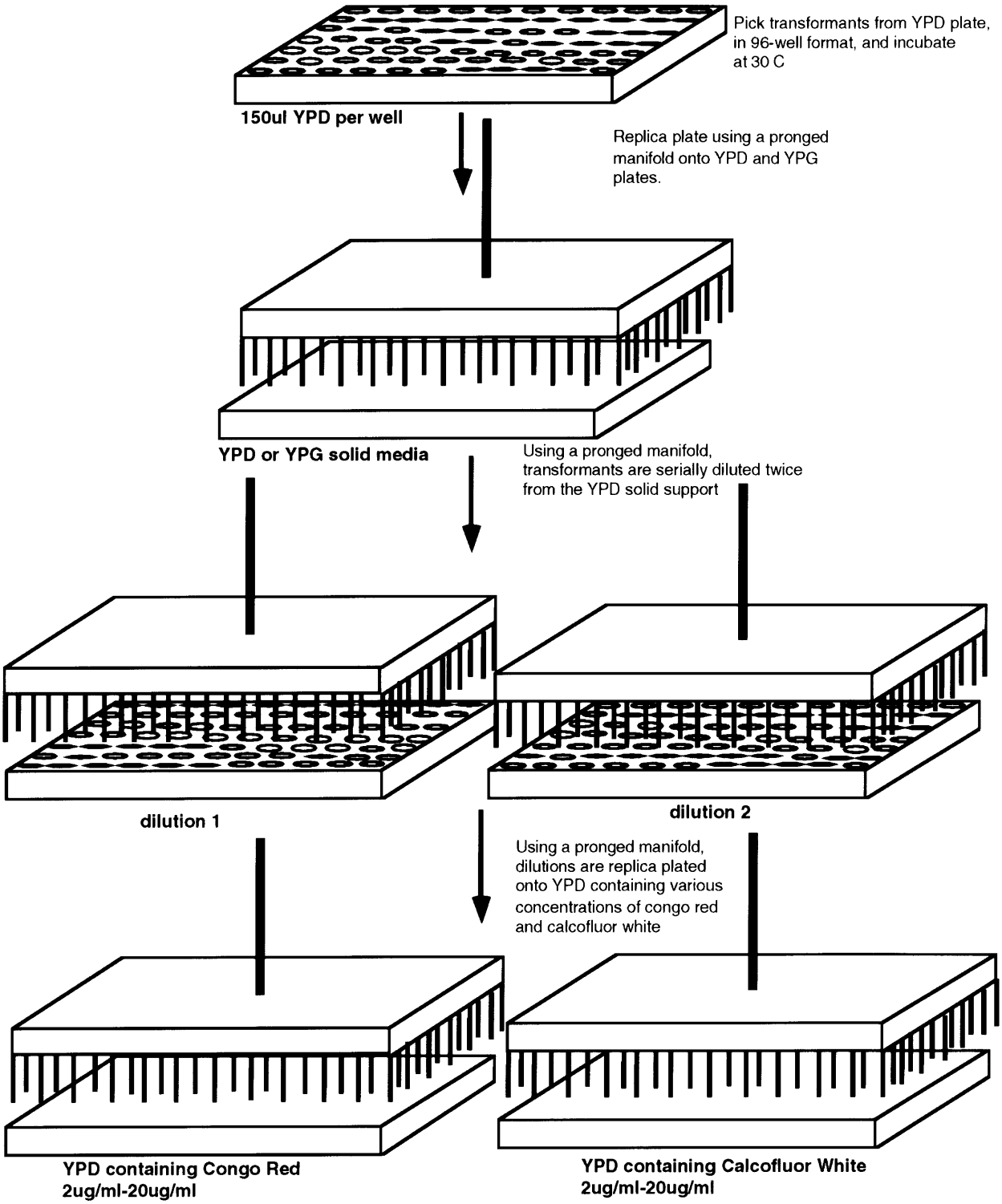
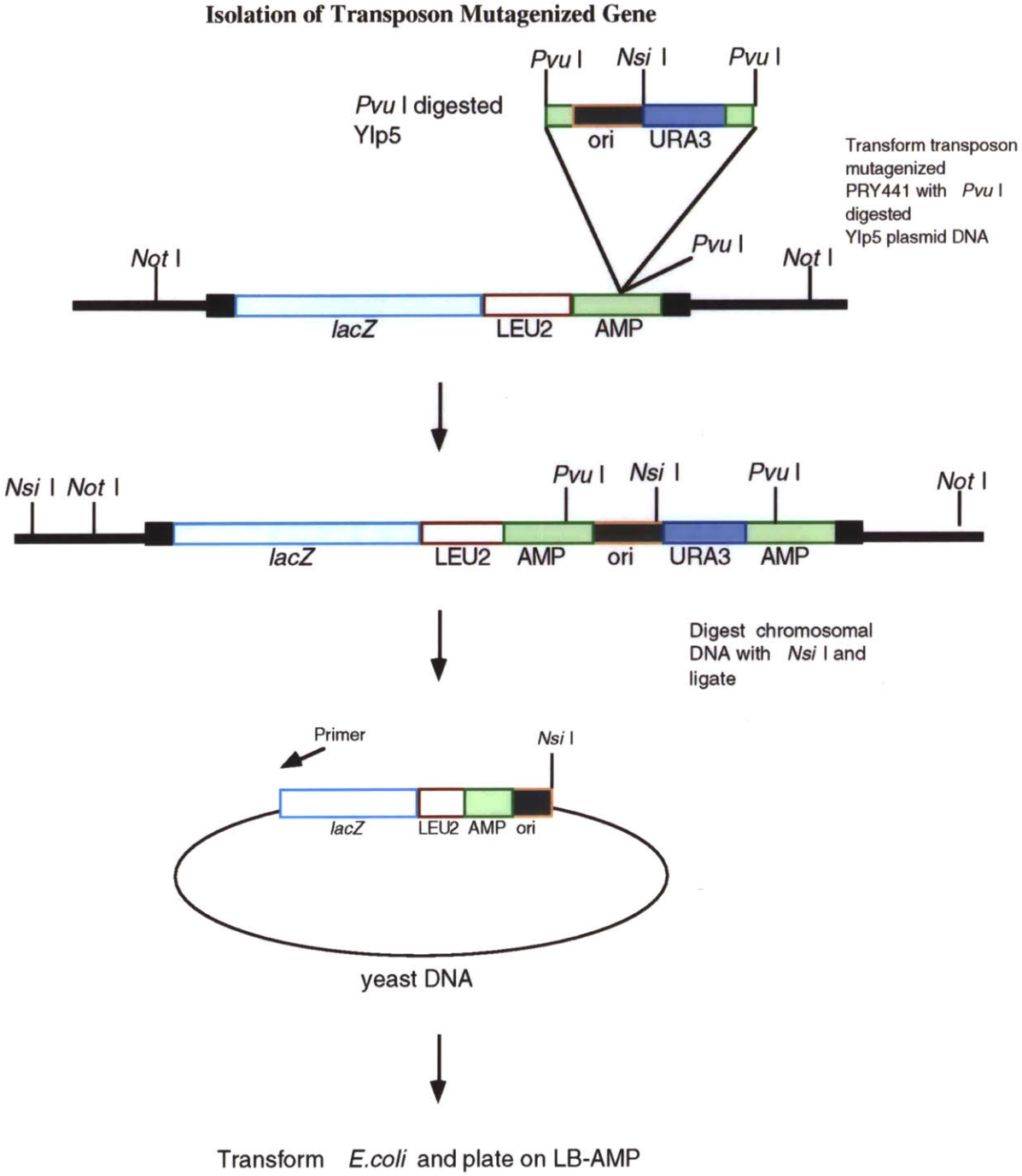


Figure 10


Isolation of Transposon Mutagenized Gene
Pvu I
Nsi I
Pvu I
Pvu I digested
Ylp5
ori
URA3
Transform transposon
mutagenized
PRY441 with Pvu I
digested
Ylp5 plasmid DNA
Not I
Pvu I
Not I
lacZ
LEU2
AMP
Nsi I
Not I
Pvu I
Nsi I
Pvu I
Not I
lacZ
LEU2
AMP
ori
URA3
AMP
Digest chromosomal
DNA with Nsi I and
ligate
Primer
Nsi I
lacZ
LEU2
AMP
ori
yeast DNA
Transform E.coli and plate on LB-AMP

E. Tables

Table 1

Cell Wall Components*

Cell Wall Component	% of wall (dry weight)	Main Linkage Types	DP[1]	Mr (kDa)	Molecules per cell x 10^6
Mannoproteins	40	α1,6 + α1,3 + α1,2		450	2.6
Glucan					
-Alkali-soluble	20	β1,3 + some β1,6	1500	243	2.5
-Alkali/acid insoluble	35	β1,3 + some β1,6	1500	243	4.3
-Alkali-insoluble, acid soluble	5	β1,3 + some β1,6	140	23	6.6
Chitin	2	β1,4			

[1]Degree of Polymerization

*The data included in this table is from Klis, 1993 and Fleet, 1991.

Table 2

Properties of the Chitin Synthases*

Enzyme	Trypsin	pH	Temp (^{0}C)	Activators	Inhibitors
CHS I	stim.	6.5	40	$Mg^{2==}$, GlcNAc, digitonin	$Co^{2=}$, polyoxin D, mikkomycins
CHS II	stim.	8.0	---	$Co^{2=}$>Mg^{2+}, GlcNAc	Polyoxin D, nikkomycins
CHS III	inhib.	8.0	25	Mg^{2+}> Co^{2+}, GlcNAc	Polyoxin D

*Data in this table from Bulawa, 1993

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II. Large Scale Identification of Genes Involved in Cell Surface Biosynthesis and Architecture in *Saccharomyces cerevisiae**

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Large Scale Identification of Genes Involved in Cell Surface Biosynthesis and Architecture in *Saccharomyces cerevisiae*

Marc Lussier,* Ann-Marie White, Jane Sheraton,* Tiziano di Paolo,* Julie Treadwell,* Susan B. Southard**, Craig I. Horenstein**, Joan Chen-Weiner**, Arthur F. J. Ram***, Johan C. Kapteyn***, Terry W. Roemer****, Dahn H. Vo,* Dana C. Bondoc,* John Hall,* Wu Wei Zhong,* Anne-Marie Sdicu,* Julian Davies*****, Frans M. Klis***, Phillips W. Robbins** and Howard Bussey***

**Department of Biology, McGill University, Montreal, Quebec, Canada, **Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, ***Institute for Molecular Cell Biology, BioCentrum Amsterdam, 1098 SM Amsterdam, The Netherlands, ****Department of Biology Yale University, New Haven, Connecticut 06520 and *****Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada*

Running head: Cell Surface Genes

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Corresponding author: Howard Bussey
Department of Biology
McGill University
1205 Dr. Penfield Avenue
Montreal, Quebec
Canada H3A 1B1

Telephone: (514) 398-6439
(514) 398-2595
Email: hbussey@monod.biol.mcgill.ca

ABSTRACT

The sequenced yeast genome offers a unique resource for the analysis of eukaryotic cell function and enables genome-wide screens for genes involved in cellular processes. We have identified genes involved in cell surface assembly by screening transposon-mutagenized cells for altered sensitivity to calcofluor white, followed by supplementary screens to further characterize mutant phenotypes. The mutated genes were directly retrieved from genomic DNA and then matched uniquely to a gene in the yeast genome database Eighty-two genes with apparent perturbation of the cell surface were identified, with mutations in 65 of them displaying at least one further cell surface phenotype in addition to their modified sensitivity to calcofluor. Fifty of these genes were previously known, 17 encoded proteins whose function could be anticipated through sequence homology or previously recognized phenotypes and 15 genes had no previously known phenotype

Determination of the *Saccharomyces cerevisiae* genome sequence focuses attention on how to make effective use of this unique resource to provide a global description of eukaryotic cell function (GOFFEAU *et al.* 1996). Strategies to determine the role of each of the approximately 6000 yeast genes, especially the 2400 of unknown function, remain unclear (DUJON 1996). Two main strategies have been proposed (OLIVER 1994, 1996). The ease of gene disruption in yeast has led to efforts to undertake the task of sequentially disrupting every gene in the genome. Such a comprehensive collection of mutants would complement the sequence and aid the study of gene function. A "genome-wide" disruption series has been started by the international yeast community and should be completed in 2-3 years (OLIVER 1996). The collection will be distributed among researchers, who will apply their own specialized phenotypic tests to the mutants.

The hierarchical classification of the many new and unknown yeast genes into families related by function constitutes a second approach (OLIVER 1994, 1996). A potential strength of this strategy is that classifying genes into functional subgroups avoids having to do detailed analysis on each and every gene in the genome. In the simplest case, only those genes within a subgroup are further analyzed by more specific tests.

Here we have made an initial attempt to identify a broad functional class of genes: those involved with the biology of the cell surface.

The cell wall is composed of the major polymers, glucan, glucomannoproteins and mannoproteins and chitin, which are synthesized and elaborated into an extracellular matrix (FLEET I991; BULAWA 1993; HERSCOVICS and ORLEAN 1993; KLIS 1994; LEHLE and TANNER 1995; VAN DER VAART *et al.* 1995) . This extracellular matrix constitutes an organelle that is dynamically engaged with the plasma membrane and the underlying secretory organelles (PRYER *et al.* 1992) along with cytoskeletal and cytoplasmic components to maintain cell integrity during growth and morphogenesis (MULHOLLAND *et al.* 1994; CID *et al.* 1995). The cell surface varies in shape and composition throughout the life of a fungal cell; in the budding of vegetative cells, in mating projection formation, in cell fusion in haploid cell conjugation, in spore wall formation following meiosis and in the specialized cell surfaces and morphogenesis seen in pseudohyphal growth (MADDEN *et al.* 1992; FLESCHER *et al.* 1993; KRON *et al.* 1994; MULHOLLAND *et al.* 1994; CHANT and PRINGLE 1995; CID *et al.* 1995). In view of the complexity of this organelle, the number of genes directly or indirectly involved in cell wall synthesis and elaboration is expected to be large. However, only a relatively small fraction of these genes have been identified and functionally characterized (KLIS 1994; RAM *et.al.* 1994; CID et al. 1995). The aim of this study is to identify, phenotypically analyze

and attempt to classify genes involved in these processes.

MATERIALS AND METHODS

Yeast strains, culture conditions and methods: All yeast manipulations were done in the AWM3CΔ630 (*MAT***a** *cir°* *leu2-3,2-112* *his3-11,3-15)* (VERNET *et al.* 1987), PRY441 *(MAT***a** *cir°* *leu2-Δ1* *ura3-52* *his3-100* *1ys2-801a* *ade2-1°* *gal3)* or PRY442 (*MATα* *cir°* *leu2-Δ1* *ura3-52* *his3-100* *lys2-801ª* *ade2-1°* *gal3)* backgrounds. Yeast cells were grown under standard conditions, (YEPD, YNB and Halvorson medium) as previously described (BROWN *et al.* 1994b). Calcofluor white solutions were either prepared fresh at 20 mg/ml and filter sterilized or were prepared at a stock concentration of 10 mg/ml in 50% ethanol and stored, in the dark, at —20° for a period of up to 1 mo. Calcofluor white containing plates were made as follows: calcofluor white solution was added to either pH 6.4 YNB agar (melted and kept at 70°) containing glucose and required supplements or to YEPD agar (melted and kept at 55°) containing glucose.

Generation of transposon-mutagenized yeast library: Haploid strains AWM3CΔ630 and PRY441 were mutagenized using transposon *Tn3::LEU2::1acZ* according to BURNS *et al* (1994) Briefly, a yeast genomic library was mutagenized in *Escherichia coli* to generate a large number of independent gene-containing transposon insertions (kindly provided by Dr. MICHAEL SNYDER) The mutated yeast DNA was then released from vector DNA by digestion with *NotI* and was transformed into the appropriate

strains using the LiAc/SS-DNA/PEG procedure (GIETZ *et al.* 1995) or the rapid transformation procedure of SONI *et al.* (1993). Yeast cells carrying the transposon as a recombinational replacement of the genomic copy with the transposon-mutagenized version were selected on synthetic minimal medium with auxotrophic supplements but lacking leucine

Southern analysis of transposon insertions: In the Tn3*::lacZ::LEU2* transposon, the *lacZ* gene is flanked on its 3' side by an *EcoRI* site. Mutant yeast genomic DNA was consequently digested with *EcoRI,* separated through a 0.8% agarose gel, transferred to a nylon membrane and hybridized with a ^{32}P-labeled-probe covering most of the *lacZ* sequence. The Tn lacZ-containing fragment detected after Southern analysis reflects a particular integration event since the other *EcoRI* site (5' from the Tn *lacZ)* is within the flanking genomic sequence. Each band visualized after autoradiography corresponds to an individual integration event.

Isolation of calcofluor white mutants: Mutagenized AWM3CΔ630 yeast cells were replica plated on YNB plates without leucine containing 20 µg/ml calcofluor white and all mutants that showed calcofluor white hypersensitivity were reverified in a plate assay according to RAM *et al. (1994).* Briefly, mutant AWM3CΔ630 cells were grown to an OD_{600} value of 0.5 and 10^{-1}, 10^{-2}, and 10^{-3} cell dilutions were

made. Three microliters of each dilution series were then spotted onto a series of YNB petri dishes containing varying amounts of calcofluor white up to 20 μg/ml. Final identification of mutants was made by scoring for growth after 48 hr at 30°. Mutagenized PRY441 yeast cells were picked and resuspended in YEPD liquid broth in a 96-well dish. Each 96-well dish contained three wells into which the parent strain (PRY441) and two predetermined mutants (one resistant and one hypersensitive) had been inoculated. The transformants were then replica plated, using a pronged manifold, to YEPD solid medium (in rectangular Nunc plates) and allowed to grow for 48 hr. The transformants were then serially diluted using a pronged manifold into 2 x 100μl ddH_2O. Each of the dilutions was then plated, using a pronged manifold onto rectangular plates containing 5, 10 or 15 μg/ml calcofluor white. For reverification, PRY441 mutants were grown overnight at 30° and then diluted to concentrations of ~1000, 100, 10, and 1 cell per μl. Five microliters of each dilution was then spotted onto plates containing 1-15 μg/ml calcofluor white. All mutants obtained showing hypersensitivity or resistance upon reverification were further analyzed.

Mating: To determine if the calcofluor white phenotype resulted from a transposon gene disruption, mutants obtained with strain PRY441 were crossed With PRY442 and the diploids were sporulated. All four

spores were analyzed for calcofluor white resistance or hypersensitivity. All mutant phenotypes segregated with the transposon insertion.

Identification of genes causing calcofluor white phenotypes: Transposon-disrupted genes causing calcofluor white phenotypes were identified by plasmid rescue and DNA sequence analysis. Individual mutant yeast cells were transformed with 50-75ng of *URA3*-based *Hpa*I-linearized pRSQ1 or *Pvu*l-linearized YIp5 plasmids using the lithium acetate procedure with sheared, denatured carrier DNA (GIETZ *et al.* 1995) or electroporation (SIMON 1993). Transformants were selected on YNB plates lacking both leucine and uracil. Yeast genomic DNA from each rescued mutant was prepared by the DTAB lysis method as previously described (GUSTINCICH *et* al 1991; BURNS *et* al 1994). The recovered genomic DNA was digested overnight by *Eco*RI (pRSQ1) or *NsiI* (YIp5) and afterwards ligated for 4 hr at 16°. The ligation mixture was transformed in *E. coli* strain DH1OB and transformants were selected on ampicillin. Plasmid DNA was prepared from individual colonies and verified by restriction digesting with *Bam*HI plus *Eco*RI (pRSQ1) or *Eco*RI alone (YIp5). Rescued vector pRQS1 results in a 3-kilobase (kb) band with additional bands coming from genomic DNA. Correct rescue of mutant genes with vector YIp5 results after digestion in diagnostic bands of 1.0 and 1.3kb. The identity of transposon-disrupted genes was made following the determination of the DNA sequence flanking the transposon insertion using

an ABI sequencer (Applied Biosystems Inc, model 373A) or manually using the dideoxy chain-termination procedure (SANGER *et al* 1977).

Computer analysis: DNA sequence and protein homology searches were conducted on the NCBI mail server using the BLAST program (ALTSCHUL *et al* 1990). DNA and protein sequence analyses were performed using the GeneWorks (Intelligenetics, Mountain View, CA) and GeneJockey (Biosoft, Cambridge, UK) software packages. Homology searches against GenBank and other major databases served to identify all known *Saccharomyces cerevisiae* genes and their homologues.

Gene disruptions: Deletional disruptions of a number of loci for verification of calcofluor white phenotypes in strain AWM3CΔ630 were made using a PCR-mediated approach (BAUDIN *et al.* 1993; WACH *et al.* 1994). In all, 15 genes suspected of causing calcofluor white phenotypes when mutated (see Tables 2-6) were entirely replaced with a DNA fragment containing a disruption module encoding the Green Fluorescent Protein and the *HIS3* gene (NIEDENTHAL *et al.* 1996). The tested genes/open reading frames (ORFs) include *YBR065c* (*ECM2*), *YKR076w (ECM4), YMR176w (ECM5), YEL030w (ECM10), YBL043w (ECM13), YHR132c (ECM14), YJR137c (ECM17), YDR125c (ECM18), YLR390w (ECMl9), YBL1O1c (ECM21), YHL030w (ECM29), BUD8, HAL5, MRE11* and *TFC1.* The DNA fragments used for each disruption were prepared by PCR using plasmid

pBM2983 as a template. In all cases, oligonucleotides used for the production of the disruption DNA fragment contained two sections a 5' region of ~50 nucleotides that corresponds either to the region immediately upstream to the start codon or to the region directly downstream of the stop codon and a 3' portion (~20 nucleotides) identical to the DNA flanking the GFP-*HIS3* module. Haploid yeast cells were transformed with PCR products. *HIS3* integrants were selected on minimal medium lacking histidine and gene disruptions were confirmed by PCR analysis (BAUDIN *et al.* 1993; NIEDENTHAL *et al.* 1996) (data not shown). Deletional disruptants were checked for calcofluor white phenotypes.

Phenotypic tests: *Mannose:glucose ratios:* The sugar composition of stationary phase mutant cells was determined as previously described (RAM *et al.* 1994).

Zymolyase sensitivity: Cultures of mutant yeast cells were grown overnight to stationary phase in YNB with all requirements or in YEPD. Cells were washed twice in water and resuspended in 10 mM Tris, pH 7.4. Approximately 1.5 x 10^7 cells were resuspended in the same buffer containing Zymolyase 20T (ICS, Montreal, Quebec) at a concentration of 3 mg/ ml. AWM3CΔ630 cell density was measured by OD_{600} at the start of the incubation and again after 1 hr. The decrease of the optical density reflects the proportion of cells that have lysed. A particular AWM3CΔ630

mutant was determined to be Zymolyase hypersensitive when the OD_{600} measured after 1 hr was <50% that of a wild type. In the case of PRY441, mutants were directly scored on plates for growth after treatment. Treated and untreated wild-type (as control) and mutant cells were serially diluted and a certain amount of each dilution series was then spotted onto YNB and YEPD petri dishes.

Hygromycin B/papulacandin B/caffeine sensitivity: Testing of mutants was similar for all three drugs Hypersensitivity or resistance was determined in the same way as for calcofluor white sensitivity (RAM *et al* 1994). Briefly, mutant cells were grown to stationary phase, diluted to an OD_{600} value of 0.5 and 10^{-1}, 10^{-2} and 10^{-3} cell dilutions were made. Three microliters of each dilution series were then spotted onto a series of YEPD petri dishes containing varying amounts of each drug, namely 50 and 100 mg/ml for hygromycin B; 1, 1.5 and 3 mg/ml for papulacandin B and 1 and 1.5 mg/ml for caffeine. Final identification of mutants was made by scoring for growth after 48 hr at 30°.

K1 killer toxin: Levels of sensitivity to K1 killer toxin were evaluated by a seeded plate assay using a modified medium consisting of 0.67% YNB, 0.0025% required amino acids, 1.0% Bacto agar, 0.001% methylene blue, 2% glucose and buffered to pH 4.7 with Halvorson minimal medium (BROWN *et al.* 1994b).

RESULTS

Transposon mutagenesis and screening for calcofluor white-hypersensitive and -resistant mutants: To identify genes involved in yeast cell surface assembly, we used transposon mutagenesis and a broad-based phenotypic screen to identify mutants. The mutated genes were retrieved from genomic DNA and identified by a short DNA sequence adjacent to the transposon tag. This procedure obviated the need for genetic complementation approaches to gene identification and enabled large numbers of new *S. cerevisiae* genes to be identified through their phenotypes. The yeast transposon library and the gene recovery and identification methodology were devised by BURNS *et al.* (1994). Similar and complementary approaches have been used by others (DANG1994; CHUN and GOEBI 1996; MOSCH and FINK 1997). To study cell wall elaboration, a primary screen was performed using calcofluor white hypersensitivity (RAM *et al.* 1994). Calcofluor white is a negatively charged fluorescent dye that binds to nascent chains of chitin and, to a lesser extent, glucan through hydrogen bonding and dipole interactions and, by preventing microfibril assembly, interferes directly with the supramolecular organization of the cell wall (ELORZA *et al.* 1983; MURGUI *et al.* 1985; RAM *et al.* 1994). A disturbed or weakened cell wall is not able to withstand drug concentrations that do not affect normal wild-type cells. Preliminary analyses using this screen identified 53 complementation

groups affecting cell wall assembly (RAM *et al.* 1994), but only 17 genes have been identified because they must be cloned by complementation (see Table 1). Two different haploid yeast strains (strains AWM3CΔ630 and PRY441) showing significantly different levels of sensitivity to the drug were used. Wild-type cells with the AWM3CΔ630 background start to be affected at calcofluor white concentrations of 20 μg/ ml and cannot grow at concentrations >30 μg/ml. Wild-type PRY441 cells are more sensitive as they cannot withstand concentrations of calcofluor white >7 μg/ml.

Approximately 9000 mutated *S. cerevisiae* cells were obtained after transformation of a yeast genomic library previously mutagenized in *E. coli* by transposon Tn3*::lacZ::LEU2* (BURNS *et al.* 1994) . To assess the extent of the mutagenesis, several verifications were performed. The proportion of disrupted yeast cells producing β-galactosidase, which depends on an in-frame insertion of the transposon into the coding region of an expressed gene and reflects the randomness of the disruption mutagenesis, was ~12% (data not shown), similar to that previously obtained (BURNS *et al.* 1994). Only five of 99 tested mutants had two independently integrated transposons in their genomes based on Southern blot hybridization analysis (data not shown). Thus, the majority of mutants possess only one insertion, a result similar to that found previously (BURNS *et al.* 1994). The

mutants were screened for calcofluor white hypersensitivity (see MATERIALS AND METHODS). Of 9000 mutants examined, 67 were stably hypersensitive to calcofluor white; 15 were more resistant to the drug than the parental wild type.

Identification of the genes causing calcofluor white phenotypes: To identify the disrupted genes, all mutants were transformed with a linearized *URA3*-containing "recovery" plasmid and the transposon-rescued mutants selected by their *LEU2* and *URA3* prototrophies. The DNA sequence adjacent to the *lacZ* gene of the transposon was obtained and the genes were identified by comparison to the complete yeast genome sequence. The transposon inserted directly in the ORF coding sequence in 79% of cases, 20% of cases in the immediate 5' upstream presumed promoter region of a gene and once *(SLN1;* see Table 3) after a stop codon in the 3' noncoding region. The mutants identified three classes of genes representing a broad spectrum of functional categories: (1) 50 genes of previously known function (61%); (2) 17 encoded proteins having homology to known proteins or possessing some known domain signature or phenotype (21 %) and (3) 15 genes (18%) were novel and of unknown function. The genes of the latter categories were serially named *ECM,* for extra cellular mutants. The identified genes were classified according to their sequence similarities and possible roles inferred (see Tables 2-6).

The proportion of genes identified in this way are similar to the proportion of known and unknown genes in the yeast genome as a whole. Thus, importantly, we are revealing novel genes with this screen, not merely uncovering previously identified genes. Of a set of 59 genes from strain AWM3CΔ630, 54 were isolated once, two genes were isolated twice *(KRE6* and *ECM15),* two were isolated three times *(TFC1* and *ECM2)* and one was isolated four times *(MRE11).* Of a set of 25 genes from strain PRY441, 22 were isolated once and three were isolated twice (*TFC1, SLG1* and *ECM34)* . Only two genes were identified in both strains (*TFC1* and *ACS1)* during this screen. Of the original set analyzed by RAM *et al.* (1994) only one, *KRE6,* was reisolated here. Thus, the calcofluor white screen is not saturated at this stage and should allow further identification of genes.

Verification of the association of calcofluor white phenotypes with identified genes: To demonstrate that the calcofluor white phenotypes were the result of the identified transposon insertions, a fraction of the genes identified in strain AWM3CΔ630 was entirely disrupted and the calcofluor white phenotype was examined. In all, 15 different genes were deleted in this way and all showed calcofluor white phenotypes similar to, or more severe than, those seen in the original mutants (see MATERIALS AND METHODS). In the case of the 20 mutants obtained in strain PRY441, phenotypic verification was carried out by

meiotic co-segregation. Tn3::*lacZ::LEU2* disruption mutants were crossed with strain PRY442 and the resulting diploids sporulated tetrad analysis revealed a 2:2 segregation of leucine prototrophy correlating with calcofluor white hypersensitivity or resistance, demonstrating that the phenotype was caused by transposon insertion. It can therefore be concluded that, for the great majority of the mutants, the calcofluor white phenotypes are the direct result of a Tn disruption.

Functional characterization by additional phenotypic tests:

The genes identified were considered to be candidates for involvement in cell surface biology. To further characterize the genes of unknown function and to better define the roles of the group of known genes in cell wall elaboration, additional phenotypic screens were performed.

Cell wall composition: The relative proportions of glucose, mannose and *N*-acetylglucosamine (GlcNAc), the three main cell wall hexoses, were determined in all mutants and 57% (47/82) of these showed an alteration in cell wall sugar ratios. These alterations could be grouped in a series from low mannose through normal wild-type ratios to low glucose, others had modified levels of GlcNAc (see Tables 2-7). Such an analysis suggests areas of function. For example, defects in genes known to affect glucose transport or glucan synthesis like *RGT2* (MARSHALL-CARLSON *et al.* 1991) (Table 3) and *KRE6* (ROEMER *et al.* 1993) (Table 2) cause reduced cell wall

glucose; while mutations in the mannosyltransferase encoding genes *KTR6* (LUSSIER *et al.* 1997) and *ALG9* (BURDA *et al.* 1996) (Table 3) cause reduced proportions of mannose. The amount of chitin in the cell wall is low and mechanisms exist to overproduce it when the cell wall is stressed or through suppression of cell wall mutations (BULAWA 1993; RAM *et al.* 1994) . A group of calcofluor white-hypersensitive mutations in 13 genes cause elevated N-acetylglucosamine levels, and there are likely to be many and differing reasons for this elevation. A smaller group of mutations in six genes cause reduced levels of GlcNAc and one of these (*lag2)* results in some resistance to calcofluor white, consistent with less chitin to bind the drug, whereas mutations in the other five genes cause hypersensitivity to calcofluor white. Two of the genes that resulted in high levels of GlcNAc when mutated *(MSNI* and *BUD8)* are involved in morphogenic processes (ESTRUCH and CARLSON 1990; ZAHNER *et al.* 1996).

Zymolyase sensitivity: Sensitivity of yeast cells to this β1,3-glucanase and protease-containing yeast lytic preparation was used to monitor changes in cell wall composition and arrangement (DE NOBEL *et al.* 1990; RAM *et al.* 1994). Possible explanations of a greater or diminished accessibility of the glucanase towards cell wall β1,3-glucans include (1) incomplete *N*- and *O*-linked polysaccharides; (2) defect in incorporation of cell wall proteins; (3) diminished levels of branched β1,3-glucan polymers.

Using this assay, 26 of 82 mutants tested showed an altered sensitivity to Zymolyase digestion compared to wild-type cells, a phenotype consistent with cell wall defects.

Hygromycin B: Fungi show limited sensitivity to aminoglycoside antibiotics like hygromycin B but yeast cells showing marked *N*-glycosylation defects are rendered sensitive to these drugs (BALLOU *et al.* 1991; DEAN 1995), but the basis for this phenotype is unclear. Twenty-five mutants showed hypersensitivity and two were found to be resistant. *VAN1* (Tables 3 and 7) is a previously recognized gene giving a hygromycin B phenotype (BALLOU *et al.* 1991; DEAN 1995). However, most of the mutants that were obtained in this screen could not be directly attributed to *N*-glycosylation defects. To extend this, we examined a set of cell wall mutants for hypersensitivity to this drug. *kre5* (MEADEN *et al.* 1990), *pmtl* and *pmt2* mutants (LUSSIER *et al.* 1995b; GENTZSCH and TANNER 1996) were all more sensitive than their isogenic parental strain; thus defects in β1,6 glucan synthesis and *O*-mannosylation also lead to hygromycin hypersensitivity (data not shown). Such strains are not generally drug sensitive, as no sensitivity was found with other antibiotics, namely, fusidic acid, emetine, a fluoroquinolone, or siomycin. Thus, sensitivity to this antibiotic constitutes a new and broad screen for cell surface defects and a wide functional variety of genes were obtained.

Papulacandin B: The glycolipid Papulacandin B is thought to be an inhibitor of β1,3-glucan synthesis. It has been postulated that it may directly hinder some components of the β1,3-glucan synthase complex (BAGULEY *et al.* 1979; KOPECKA 1984; RAM *et al.* 1994) or inhibit incorporation of β1,3-glucans into the molecular organization of the extracellular matrix (FONT DE MORA *et al.* 1993). Mutations in 22 genes resulted in Papulacandin B-hypersensitivity phenotypes, though these showed no correlation with altered levels of glucose, mannose or GlcNAc as was previously found (RAM *et al.* 1994). Consistent with this, hypersensitivity to this drug could not be specifically linked to cell wall β1,3-glucan defects (RAM *et al.* 1994) . In the RAM *et al.* (1994) calcofluor mutant collection, three mutated yeast strains *(cwh26, cwh32* and *cwh53-1)* additionally showing Papulacandin B hypersensitivity had their causative gene isolated (see Table 1): *FKSI* encodes a subunit of the 1,3-β-D-glucan synthase (RAM *et al.* 1995) and can obviously be directly linked to this type of defect. However, the two other identified genes *(VMAI* and *VPS16;* see Table 1) encode vacuolar proteins (CONIBEAR and STEVENS 1995). Both our screen and that of RAM *et al.* (1994) indicate that Papulacandin B hypersensitivity is not β1,3-glucan specific, but permits detection of a broad range of cell wall defects.

K1 killer toxin: K1 killer yeast strains secrete a small pore-forming toxin that requires a cell wall receptor for function (BUSSEY 1991). Killer resistant mutants have been found to be defective in β1,6-glucan and in O-mannosylation, indicating that the *in vivo* receptor includes these polymers (BOONE *et al.* 1990; HILL *et al.* 1992; ROEMER *et al.* 1993; GENTZSCH and TANNER 1996). Five genes, all known, were identified here with resistance phenotypes, three in expected classes *(ALG9, KRE2, KRE6,* two unexpected, *IMP2 (DONNINI et al.* 1992) and *PAS8* (VOORN-BROUWER *et al.* 1993), with other wall phenotypes. A larger class of eight mutants led to killer toxin hypersensitivity and these have never been previously screened for in a systematic way. Mutations leading to wall defects that retain a wall receptor can lead to hypersensitivity. Disruption of *PKCI* (ERREDE and LEVIN 1993) with a wall with reduced amounts of all polymers or *PBS2* lead to hypersensitivity (ROEMER *et al.* 1994; JIANG *et al.* 1995); this may be a good indicator of wall changes. Three known genes associated with this phenotype are regulatory, the five novel ones are associated with a range of additional wall phenotypes.

Caffeine: This drug is an inhibitor of cAMP phosphodiesterases (PARSONS *et al.* 1988). Several mutants involved in growth control and in the *PKCI-MPKI* signal transduction pathway show increased sensitivity to caffeine (COSTIGAN *et al.* 1992; PARAVICINI *et al.* 1992; POSAS *et al.* 1993;

RAM *et al.* 1994). This phenotype is loosely indicative of a defect in regulation/signal transduction, and all mutants were tested for growth in the presence of this drug. Caffeine sensitivity is a common phenotype among this calcofluor collection (24%), with five resistant and 15 hypersensitive mutants. Among the 15 known genes identified, nine can reasonably be termed regulatory. *MKSI,* which acts as a negative regulator of the RAS-cAMP pathway (MATSUURA and ANRAKU 1993), was picked up in the screen.

Morphology: Because the cell wall determines cellular architecture, cells of the different mutants were evaluated for altered morphology. Four mutants were found to possess an abnormal morphology when compared to the wild type. All four mutants *(ecm2, ecm5, ecml9* and *ecm20)* showed a similar morphology: cells were enlarged, having a cellular volume of up to four times greater than wild-type cells, and had large drooping buds with an elongated neck (data not shown). Interestingly, these mutants have been found to be pleiotropic possessing many of the tested phenotypes.

DISCUSSION

The mapping and sequencing phase of the yeast genome is complete, and work now focuses on functional analysis of the component genes. Systematic functional analysis of this magnitude breaks new ground in genomics. For yeast genes of unknown function, and for many of the already known genes, definitive roles remain to be determined. Here we show that broad "genome-wide" screens are possible and constitute an immediate approach to functional analysis. Transposon mutagenesis/gene recovery technology coupled with a calcofluor white screen is an efficient approach to identifying genes with mutations that cause defects in all the major cell surface polymers.

Classification by sequence similarity to genes of known function: The genes sampled represent a rich cross section of the yeast genome and include known genes, some not previously suspected to be involved in cell surface biology, and genes of unknown function found by systematic sequencing. Initial characterizations of the mutant gene collection by sequence similarity, from which function can often be inferred, has permitted their categorization into broad and sometimes overlapping classes that are outlined illustratively in Tables 2-6.

Known genes related to cell wall assembly: In some cases, the transposon integrated in genes of known function with a clear role in cell wall elaboration or structure (see Table 2). Other genes can reasonably be

related to processes impinging on the cell surface (see Table 3). These include genes that function in the secretion pathway or that are involved in maintaining cellular integrity or required in morphogenesis or for the pseudohyphal cell type.

Known genes with an unanticipated involvement in the cell surface: The unexpected association of some known cellular genes with cell wall phenotypes (Table 4) emphasizes the value of genome-wide screens to define function and to examine global aspects of regulation in the yeast cell. Such genes perform a wide range of roles, ranging from involvement in metabolism, mitochondrial function, transcription, translation and DNA repair. Many of the effects seen in these mutants are likely indirect. For example, a yeast cell that transcribes or translates incorrectly because of enzymatic machinery problems may produce defective proteins and ultimately the cell wall and other cellular organelles will have a modified composition. However, one must not overlook possible regulatory associations between cellular pathways and cell wall synthesis and assembly. A prime candidate for such an association is *MREll* (Table 4); it classically encodes a DNA repair protein (JOHZUKA and OGAWA 1995) and appears incongruous here. Mutations in the gene were isolated independently four times and have a range of strong cell surface phenotypes. While we do not understand what is happening in *mrell* mutants, we can draw on our knowledge of prokaryotes for precedents.

DNA synthesis and repair and the SOS response are known to lead to cell surface morphological changes, osmoresistance and filamentous growth in *E. coli* and *Bacillus subtilis* (ENNIS *et al.* 1993; RUZAL *et al.* 1994). Further work will be required to establish if we have uncovered an analogous "global" response in yeast.

Another possible example of a global regulatory response is illustrated by mitochondrial defects that appear to perturb the yeast cell surface. *IFM1, SMP2* and *COX1* are all nuclear petite genes (VAMBUTAS *et al.* 1991; IRIE *et al.* 1993; TZAGOLOFF *et al.* 1993) with cell surface phenotypes. Again this relationship seems unexpected. However, there is an earlier literature on this theme that, perhaps because of an underlying lack of an explanatory paradigm, has been overlooked (EVANS *et al.* 1980; WILKIE *et al.* 1983). Our results independently suggest that there may be some regulatory link between mitochondrial function and the cell surface. These results are surprising and can be viewed in two ways. One can dismiss them as nonspecific or indirect or indicative of the bluntness of the primary calcofluor screen. Alternatively, mutations in these genes, which do give strong cell wall phenotypes consistent with the screen working, are identifying unanticipated interactions of these genes. Finding new roles for established genes is bound to be a controversial activity but is likely to be an important and general outcome of genome wide functional screens.

ORFs for which there is limited functional information or no known function: Some ORFs gave a match defining the biochemical class but not the specific function of the gene (Table 5). In a limited number of cases, the biochemical role of the gene is unknown but some superficial information about function has been reported. ORFs of totally unknown function that were discovered through genome sequencing, the so-called single Orphan genes (DU JON 1996), are listed in Table 6, some of these have homology with another yeast hypothetical protein or with an ORF from some other organism.

Attempts at a hierarchical classification through cell surface phenotypes: To try to classify further the calcofluor white collection, a number of additional screens were undertaken. Simple wall-related phenotypes were scored to aid a progression to specific analysis of function in extracellular matrix assembly.

Cell wall hexose levels: Examination of the amounts of cell wall polymer sugars offers a powerful way to sort mutants, as a reduced level of a component sugar likely indicates a defect leading to reduced synthesis of the relevant polymer. Such a classification formed the primary basis for classifying the original calcofluor white hypersensitive collection (RAM *et al.* 1994). However, there are some caveats and limitations in this approach that would have to be further examined in working with individual mutants. These reflect the fact that only sugar ratios and not absolute

amounts of polymers have been determined. Thus if the level of all polymers falls, the ratio of the sugars may not change. A significant number of mutants (43%) do not show an obvious change in monomer ratios, but some may have suffered pleiotropic effects leading to a global reduction in polymer levels or may have more modest defects in the cell wall (like the Kre2p mannosyltransferase (HAUSLER and ROBBINS 1992; HAUSLER *et al.* 1992; HILL *et al.* 1992; LUSSIER *et al.* 1995a) or may have no wall defect at all. If the level of the mannose or glucose polymers rise, this will be interpreted using the ratio method as a fall in the level of the other polymer. This formal possibility seems less likely, simply because of the large amounts of mannose and glucose already present in the wild-type cell wall and the consequent difficulty of their overproduction to an extent required to significantly distort the mannose:glucose ratio.

Further screens for wall phenotypes: A range of additional phenotypic screens using drugs or proteins/enzymes that affect the cell surface or its regulation have been made on the mutant collection. These attempted to confirm or extend the data from the calcofluor and sugar ratio screens into more specific functional subclassifications. The results are shown in Tables 2-7. This hierarchical classification was only of limited success. A major shortcoming that emerged was the empirical nature of many of the tests for cell surface defects, with many of the drugs

identifying a broad range of genes affecting many cell surface processes and limiting a useful hierarchical classification.

Of the original calcofluor mutations in 82 genes, 47 caused some obvious change in polymer sugar ratios. Mutations in a further 18 genes resulted in a phenotype with respect to at least one of these additional tests for a wall phenotype. Thus mutations in 65 of 82 genes (79%) caused some additional wall phenotype beyond that of altered calcofluor sensitivity. This high proportion attests to the value of calcofluor white as a reliable primary cell surface screen and is consistent with the earlier work (RAM *et al.* 1994). However, the full extent of calcofluor white toxicity is not known, and it is possible that the mutations in the 17 remaining genes do not result in cell surface defects. For example, one could imagine that not all of the drug remains extracellular and that defects in removing intracellular calcofluor white could lead to hypersensitivity.

Perspectives: This large scale screen has certain inherent limitations; some have been raised, two others are worth mentioning: lethal phenotypes will be missed as haploid strains were used and a proportion of mutated genes may not cause phenotypes because they are members of one of the large number of yeast gene families (DU JON 1996; GOFFEAU *et al.* 1996). In some cases individual members of a group of related genes have been identified, for example, *KRE2* and *KTR6* (LUSSIER

et al. 1996, 1997). In other cases, this would be unlikely, for example, the seven members of the Pmtp protein: *O*-mannosyltransferase are highly redundant and calcofluor phenotypes were only seen in cells bearing at least two disruptions (GENTZSCH and TANNER 1996) .

The genes found have been categorized according to their respective phenotypes (Table 7). The wide range of gene categories and phenotypes obtained reemphasize that cell surface synthesis and its integration with cellular growth and division is complex, with regulation of the individual polymers and likely some global overall sensing and control. Extracellular matrix synthesis has constraints, temporal in the cell cycle and spatial in cell architecture (KLIS 1994; CID *et al* 1995; IGUAL *et al.* 1996). In addition there is much physiological evidence that environmental conditions such as nutrient and carbon source, temperature and the medium osmoticum affect the composition of the cell wall (KLIS 1994; CID *et al.* 1995). Recently several major signal transduction cascades have been found to regulate the cell surface. Known components include the protein kinase C cascade (PARAVICINI *et al.* 1992; ERREDE and LEVIN 1993; IGUAL *et al.* 1996), the osmotic sensing *HOG* pathway (SCHULLER *et al* 1994; JIANG *et al.* 1995), a two component regulatory system with the *SKN7* transcription factor as a receiver module (BROWN *et al.* 1994a), and the calcium-modulated protein phosphatase, calcineurin, has been implicated in β-glucan synthesis (GARRET-ENGELE *et al.* 1995). Other protein kinases

and phosphatases identified here also have wall effects. It is likely that these different pathways are coordinated and that cell surface biosynthesis and assembly is controlled at many levels, from transcriptional regulation to the cell wall itself.

Large-scale functional studies on sequenced genomes are in their infancy. Exploratory studies like this one are informative but point up both strengths and weaknesses in the approach. Detailed functional analysis of the identified genes will be longer term and will involve the participation of yeast specialists. This will require access by the community to the large body of data on the genes. To assist this process we will place our information on the identified genes in the major databases.

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F. Tables

Table 1 Identified *CWH* genes

CWH#	Gene/ORF	Function
1	*MNN9*	Required for *N*-linked outer chain synthesis (R. Zufferey and M. Aebi, pers. comm.)
2	*VRG1*	Involved in orthovanadate resistance and protein glycosylation (R. Zufferey and M. Aebi, pers. comm.)
4	*GPI1*	Involved in GPI anchor synthesis (J. Vossen and F. Klis, unpubl. data)
6	*GPI3*	*N*-acetylglucosaminyltransferase required for GPI anchor synthesis
8	*YGR036c*	Unknown; similarity to *T. denticola* phosphatase; 239 aa (M. van Berkel and F. Klis, unpubl. data)
13	*ERD1*	Required from retention of ER proteins (A. Ram, R. Sanjuan and F. Klis, unpubl. data)
17	*URE2*	Nitrogen catabolite repression regulator (R. Montijn, S. Brekelmans and F. Klis, unpubl. data)
26	*VMA1*	Vacuolar H^+-ATPase catalytic subunit (A. Ram, R. Sanjuan and F. Klis, unpubl. data)
30	*NRK1*	Ser/Thr kinase that interacts with Cdc31p (J. Vossen and F. Klis, unpubl. data)
32	*VPS16*	Required for vacuolar protein sorting (G. Paravicini, pers. comm.)
36	*YCL007c*	Unknown; 130 aa (M. van Berkel and F. Klis, unpubl. data)
41	*CWH41*	ER protein involved in β1,6-glucan assembly
47	*PTC1*	Protein Ser/Thr phosphatase 2c
48	*KRE6*	Golgi protein involved in β1,6-glucan synthesis
50	*PLC1*	PI-specific phospholipase C (A. Ram, R. Sanjuan and F. Klis, unpubl. data)
52	*GAS1*	Involved in β1,3-glucan crosslinking
53	*FKS1*	β1,3-glucan synthase component

Table 2a Genes directly involved in cell surface assembly

Gene/ORF [a] (synonyms)	CFW [b] Phenotype	Chr	Tn [c] insertion	Function/Homology/Domain [d]	Additional [e] Phenotypes
KRE6 *CWH48* *YPR159w*	HS	XVI	CDS (2)	Golgi protein involved in β-1,6 glucan synthesis	M/G ↑ zymolyase-hs killer-rs
KRE2 *MNT1* *YDR483w*	HS	IV	promoter	Golgi α1,2-mannosyltransferase	zymolyase-hs papulacandin-hs killer-rs
*CWP2** *YKL096w-a*	HS	XI	CDS	cell wall mannoprotein	zymolyase-hs papulacandin-hs caffeine-hs
DIT2 *CYO56* *CYP56* *YDR402c*	RS	IV	promoter	belongs to the cytochrome P450 family; involved in spore wall maturation	M/G ↓

a - mutant genes were obtained in strain AWM3CΔ630, or in strain PRY441 where indicated with an asterisk (*)
- † = mutant genes obtained in both strains

b - HS = hypersensitive; RS = resistant

c - CDS = coding sequence; (#) = number of times a given gene was obtained; insertion was considered in promoter when Tn inserted in a 300 bp region just 5' from ATG of coding sequence

d - for more information consult major on-line databases such as MIPS, SGD and YPD

e - M/G = mannose:glucose ratios

Table 2b Genes plausibly related to the cell surface

Secretory Pathway

Gene/ORF (synonyms)	CFW Phenotype	Chr	Tn Insertion	Function/Homology/Domain	Additional Phenotypes
KTR6 *YPL053c*	HS	XVI	CDS	member of the *KRE2/MNT1* α1,2-manno-syltransferase gene family	M/G ↓ hygromycin-hs
MAS5 *YDJ1* *YNL064C*	HS	XIV	CDS	involved in protein import into ER and mitochondria; highly similar to *E. coli* DnaJ	
*VAN1** *VRG7* *YML115c*	HS	XIII	CDS	vanadate resistance protein; mutants show altered pattens of phosphoproteins and have defective glycosylation; may be involved in retention of enzymes in the ER or Golgi	M/G ↓ zymolyase-hs hygromycin-hs
*ALG9** *YNL219c*	RS	XIV	CDS	involved in the step-wise assembly of lipid-linked oligosaccharides in *N*-linked glyco-sylation; multiple TMD protein	M/G ↓ killer-rs caffeine-hs

Morphogenesis

Gene/ORF (synonyms)	CFW Phenotype	Chr	Tn Insertion	Function/Homology/Domain	Additional Phenotypes
MSN1 *FUP1* *PHD2* *YOL116w*	HS	XV	CDS	transcriptional activator for genes regulated through SNF1p (a multicopy suppressor of invertase defect in *SNF1* mutants); required for pseudohyphal form	M/G ↑ GlcNAc ↑ hygromycin-hs papulacandin-hs caffeine-rs
BUD8 *YLR353w*	HS	XII	promoter	required for bipolar budding	GlcNAc ↑ zymolyase-hs caffeine-rs
*BUD4** *YJR092w*	RS	X	CDS	required for formation of axial but not bipolar budding	
PPH22 *YDL188c*	RS	V	CDS	protein serine/threonine phosphatase; involved in cell integrity and morphogenesis	
*DFG16** *YOR030w*	RS	XV	CDS	involved in invasive growth upon nitrogen starvation; 619 aa; probable multiple TMD protein	M/G↓

Regulatory/ Signal Transduction

Gene/ORF (synonyms)	CFW Phenotype	Chr	Tn Insertion	Function/Homology/Domain	Additional Phenotypes
HAL5 *YJL165c*	HS	X	CDS	protein kinase involved in salt tolerance and pH sensitivity; high homology to C-terminus of Ykl168p	M/G↓ hygromycin-hs
*YCK2** *YNL154c*	HS	XIV	promoter	casein kinase I isoform; mutants have increased salt sensitivity and show defects in morphogenesis; TMD	M/G↓ papulacandin-hs
*SLN1** *YPD2* *YIL147c*	HS	IX	67 bp after stop codon	two-component signal transducer; functions in the high osmolarity signal transduction pathway	hygromycin-hs caffeine-hs
*SSK2** *YNR031c*	RS	XIV	CDS	MAP kinase kinase kinase of the high osmolarity signal transduction pathway	caffeine-rs
MKS1 *YNL076w*	HS	XIV	CDS	negative regulator of RAS-cAMP pathway; involved in carbohydrate utilization regulation	M/G↑ GlcNAc↑ killer-hs caffeine-rs
*SNF3** *YDL194w*	HS	IV	CDS	high-affinity glucose transporter; can play a positive or negative role in glucose transport; multiple TMD protein	zymolyase-hs hygromycin-hs papulacandin-hs caffeine-hs
RGT2 *YDL138w*	HS	IV	CDS	involved in regulation of glucose transport; homology with glucose transport proteins (e.g. SNF3); multiple TMD protein	M/G↑ GlcNAc↑
EFF2 *YML048w*	HS	XIII	promoter	defective in glucose repression; TMD	M/G↓ GlcNAc↑ hygromycin-hs
*ROM2** *YLR371w*	HS	XII	CDS	putative GDP-GTP exchange protein for Rho1p which is involved in regulation of β1,3-glucan synthesis	caffeine-hs
SWI6 *YLR182w*	HS	XII	CDS	involved in cell cycle regulation and in controlling the expression of some genes involved in cell wall biosynthesis	

Table 2c Genes not previously related to the cell surface

Metabolism

Gene/ORF (synonyms)	CFW Phenotype	Chr	Tn Insertion	Function/Homology/Domain	Additional Phenotypes
URA7 *YBL039c*	HS	II	CDS	CTP synthase 1; last step in pyrimidine biosynthesis pathway; activated by GTP and inhibited by CTP; TMD	M/G ↓
ACS1 † *YAL054c*	HS	I	CDS	acetyl-CoA synthetase (acetate-CoA ligase)	zymolyase-hs
GDH3 *YAL062w*	HS	I	CDS	NADP-glutamate dehydrogenase 3	
ORD1 *YKL184w*	HS	XI	promoter	ornithine decarboxylase	M/G ↑ zymolase-hs
FOX2 *YKR009c*	HS	XI	promoter	bifunctional β-oxidation peroxisomal protein	M/G ↑ hygromycin-hs
*SPE3** *YPR069c*	RS	XVI	CDS	putrescine aminopropyltransferase (spermidine synthase)	
*CIS2** *YLR299w*	RS	XII	CDS	gamma-glutamyltransferase homolog	
ARG7 *YMR062c*	RS	XIII	CDS	acetylornithine acetyltransferase	

Mitochondrial related

Gene/ORF (synonyms)	CFW Phenotype	Chr	Tn Insertion	Function/Homology/Domain	Additional Phenotypes
*IMP2'** *YIL154c*	HS	IX	CDS	nuclear gene controlling the mitochondrial dependence of galactose, raffinose and maltose utilization; TMD	zymolyase-rs hygromycin-hs killer-rs caffeine-hs
IFM1 *YOL023w*	HS	XV	CDS	mitochondrial translation initiation factor 2	zymolyase-hs papulacandin-hs
SMP2 *YMR165c*	HS	XIII	CDS	null mutant has increased plasmid stability and respiration-deficient phenotype	M/G ↑ GlcNAc↑ killer-hs
COX11 *YPL132w*	HS	XVI	CDS	required for cytochrome oxidase assembly	
PEL1 *YCL004w*	HS	III	promoter	CDP diacylglycerol-serine O-phosphatidyl-transferase; required for survival of petite mutants	GlcNAc↓ zymoylase-hs papulacandin-hs caffeine-hs

Gene/ORF (synonyms)	CFW Phenotype	Chr	Tn Insertion	Function/Homology/Domain	Additional Phenotypes
MRE11 *YMR224*	HS	XIII	CDS (4)	DNA repair protein; member of the *RAD52* epistasis group; required for double-strand repair and meiotic recombination	M/G↑ GlcNAc↓ papulacandin-hs killer-hs
HEL1 *YER176w*	HS	V	promoter	DNA helicase I	M/G↑ hygromycin-hs papulacandin-hs
*RAD23** *YEL037c*	RS	V	CDS	nucleotide excision repair protein	
MEC3 *YLR228c*	HS	XII	promoter	checkpoint protein required for arrest in G2 after DNA damage	M/G↓ caffeine-hs
RNH1 *YMR234w*	HS	XIII	promoter	ribonuclease H; degrades specifically RNA-DNA hybrids	M/G↑ hygromycin-hs papulacandin-hs caffeine-hs
PAN3 *YKL025c*	RS	XI	CDS	subunit of the Pab1p-dependent poly (A) nuclease; similarity to *C. elegans* hypothetical protein ZK632.7	
SRD2 *YPL021w*	HS	XVI	CDS	homolog of Srd1p which affects pre-rRNA processing	M/G↓
HCA4 *YJL033w*	HS	X	CDS	DEAD and DEAH box families ATP-dependent RNA helicase	M/G↓ zymolyase-hs

RNA polymerase III

Gene/ORF (synonyms)	CFW Phenotype	Chr	Tn Insertion	Function/Homology/Domain	Additional Phenotypes
TFC1 † *YBR123c*	HS	II	promoter (1) CDS (4)	95 kDa subunit of TFIIIC (TAU); mediates tRNA and 5s RNA gene activation by binding to intragenic promoter elements	M/G ↓ zymolyase-hs
RPC34 *YNR003c*	HS	XIV	CDS	DNA-directed RNA polymerase (III) chain	M/G ↓ hygromycin-hs

Others

Gene/ORF (synonyms)	CFW Phenotype	Chr	Tn Insertion	Function/Homology/Domain	Additional Phenotypes
YEF3 *TEF3* *EFC1* *YLR249w*	HS	XII	CDS	translation elongation factor eF-3; stimulates EF-1 alpha-dependent binding of aminoacyl-tRNA by the ribosome; requirement for EF-3 is unique to fungi; member of ATP-binding cassette (ABC) family	GlcNAc↑ zymolyase-hs
STF2 *YGR008c*	HS	VII	promoter	ATPase stabilizing factor	M/G ↓
*PAS8** *YNL329c*	RS	XIV	CDS	peroxisomal assembly protein	papulacandin-hs killer-rs
LAG2 *YOL025w*	RS	XV	CDS	determines yeast longevity; TMD	GlcNAc↓

Table 3 Genes of unknown function having a recognized signature, sequence similarity or a previously known phenotype

ECM#	Gene/ORF (synonyms)	CFW Phenotype	Chr	Tn Insertion	Function/Homology/Domain	Additional Phenotypes
	DCG1 *YIR030c*	HS	IX	CDS	unknown; transcript level sensitive to nitrogen-catabolite repression; TMD	GlcNAc↓ zymolyase-hs caffeine-hs
	*SLG1** *YOR008c*	HS	XV	CDS (2)	unknown; weak similarity to Ynl283p and *L. mexicana* Imsap2 gene (secreted acid phosphatase 2); 378 aa	M/G↓ hygromycin-hs papulacandin-hs caffeine-hs
10	*YEL030w*	HS	V	CDS	heat shock protein signature; 644 aa (70 kD); similarity to Pmr1p and Ens1p	M/G↑ hygromycin-hs
14	*YHR132c*	HS	VIII	CDS	similarity to zinc carboxypeptidase family; 430 aa; TMD	M/G↑ hygromycin-hs papulacandin-hs
15	*YBL001c*	HS	II	CDS (2)	unknown, 104 aa, partial homology to *S. xylosus* glucose kinase	hygromycin-hs
17	*YJR137c*	HS	X	CDS	putative sulfite reductase (ferredoxin); 1442 aa; homology to yo72h02.s1 *Homo sapiens* cDNA clone 1	zymolyase-hs hygromycin-hs
18	*YDR125c*	HS	IV	CDS	unknown; 453 aa; some similarity to aromatic hydrocarbon catabolism esterase; similarity to hypothetical protein Ylr099p (Ch XII)	
31	*YBR176w**	HS	II	CDS	strong similarity to *E. coli* 3-methyl-2-oxobutanoate hydroxymethyltransferase; 312 aa	M/G↑ hygromycin-hs papulacandin-hs

ECM#	Gene/ORF (synonyms)	CFW Phenotype	Chr	Tn Insertion	Function/Homology/Domain	Additional Phenotypes
16	*YMR128w*	HS	XIII	CDS	DEAD and DEAH box families ATP-dependent helicase signature; 1267 aa	GlcNAc↑ zymolyase-hs hygromycin-hs papulacandin-hs
20	*YGR195w*	HS	VII	CDS	unknown; 256 aa; TMD; 22% identity to *E. coli* ribonuclease over 195 aa	M/G↑ GlcNAc↑ hygromycin-hs papulacandin-hs killer-hs caffeine-hs abnormal morphology
25	*YJL201w*	HS	X	CDS	unknown; 599 aa; promoter has a consensus sequence for factor Abf1p	
30	*YLR436c**	HS	XII	CDS	unknown; 1274 aa; probable multiple TMD protein; has phosphopantetheine attachment site	M/G↓
27	*YJR106w*	HS	X	CDS	unknown; 725 aa; weak similarity to Na+/H+ antiporter; probable multiple TMD protein	papulacandin-hs
39	*YNR030w**	RS	XIV	CDS	unknown; 551 aa; probable multiple TMD protein; weak similarity to Smp3p	papulacandin-hs
33	*YBR078w**	HS	II	CDS	unknown; strong similarity to sporulation specific Sps2p and to Ydr055p; 468 aa	M/G↓ zymolyase-hs hygromycin-hs killer-hs

ECM#	Gene/ORF (synonyms)	CFW Phenotype	Chr	Tn Insertion	Function/Homology/Domain	Additional Phenotypes
5	*YMR176w*	HS	XIII	CDS	unknown; 1411 aa; some similarity to SW:X169_Human; containsATP/GTP-binding site motif A	M/G↑ GlcNAc↑ hygromycin-hs caffeine-hs abnormal morphology
8	*YBR076w*	HS	II	CDS	unknown; 339 aa; similarity to surface antigens from trophoblast endothelial-activated lymphocytes.	killer-hs hygromycin-rs

Table 4 Genes of totally unknown function

With homology to other unknown genes

ECM#	Gene/ORF (synonyms)	CFW Phenotype	Chr	Tn Insertion	Function/Homology/Domain	Additional Phenotypes
3	*YOR3165w*	HS	XV	CDS	unknown; 614 aa; probable multiple TMD protein; highly similar to Ynl095p	
4	*YKR076w*	HS	XI	promoter	unknown; 370 aa; homology to hypothetical protein Ygr154p	GlcNAc↑
21	*YBL101c*	HS	II	CDS	unknown; 1077 aa; similarity to Ypr030p	GlcNA↓
29	*YHL030w*	HS	VIII	CDS	unknown; 1868 aa; probable multiple TMD protein; similarity to *C. elegans* unknown protein D2045.2	zymolyase-hs hygromycin-rs
34	*YHL043w**	HS	VIII	CDS (1) promoter (1)	unknown; similarity to subtelomeric-encoded proteins such as Ykl219p, Ybr302p, Ycr007p, Yhl048p, Ynl336p; 2 putative TMDs; 170 aa	

Orphan genes

ECM#	Gene/ORF (synonyms)	CFW Phenotype	Chr	Tn Insertion	Function/Homology/Domain	Additional Phenotypes
1	*YAL059w*	HS	I	CDS	unknown; 212 aa; TMD	
9	*YRK004c*	HS	XI	CDS	unknown; 292 aa; TMD	papulacandin-hs
12	*YHR021w-a*	HS	VIII	promoter	unknown; 151 aa; TMD	zymolyase-hs
19	*YLR390w*	HS	XII	CDS	unknown; 112 aa; TMD	M/G↑ GlcNAc↑ zymolyase-hs hygromycin-hs killer-hs abnormal morphology
37	*YIL146c*	RS	IX	CDS	unknown; 529 aa; TMD	M/G↓ zymolyase-hs caffeine-rs
7	*YLR443w*	HS	XII	CDS	unknown; 448 aa; probable multiple TMD protein	GlcNAc↓ zymolyase-hs
2	*YBR065c*	HS	II	CDS (3)	unknown; 364 aa	zymolyase-hs hygromycin-hs papulacandin-hs killer-hs caffeine-hs abnormal morphology
11	*YDR446w*	HS	IV	CDS	unknown; 302 aa	zymolyase-hs hygromycin-hs papulacandin-hs

Orphan genes (cont'd)

ECM#	Gene/ORF (synonyms)	CFW Phenotype	Chr	Tn Insertion	Function/Homology/Domain	Additional Phenotypes
13	*YBL043w*	HS	II	CDS	unknown; 257 aa	GlcNAc↑ zymolyase-hs papulacandin-hs caffeine-hs
26		HS	VIII	promoter	unknown; 30 bp from ATG of previously unidentified small ORF (51 aa) between *SCH9* and *SKN7*	

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H. Appendix

Tables

Figures

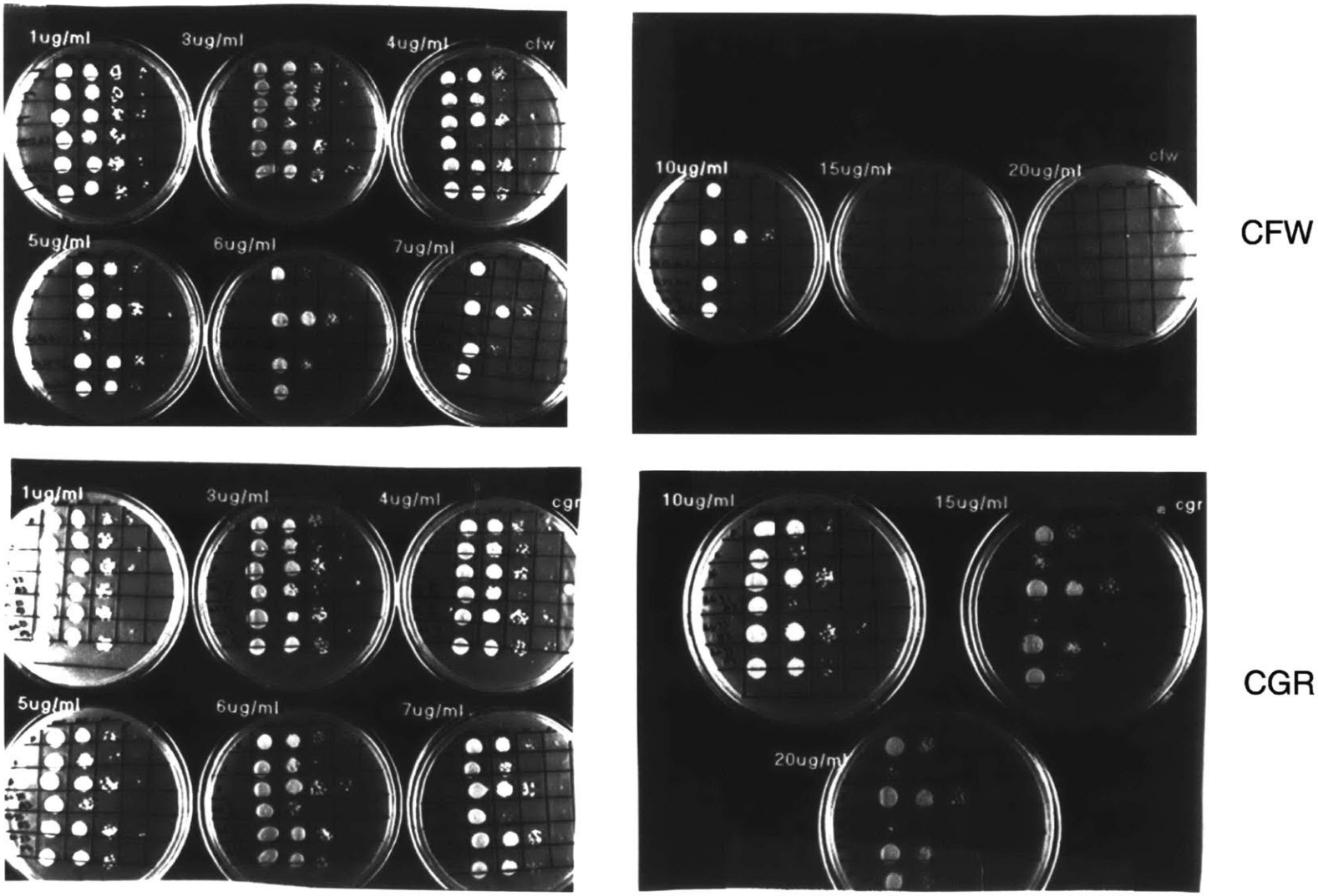
Table 1

Carbohydrate Analysis of a Subset of the Cell Surface Assembly Mutants

Strain	GlcNAc	Glc	Man	M/G ratio	CFW/CGR	Additional Information
PRY441	1.6	29.3	69.1	2.22	7ug/ml	Parent Strain
2-F11	2.4	35.5	62.1	1.75	6ug/ml	chr XII, in ORF, U21094
2-H12	3.2	28	68.8	2.34	8ug/ml	chr XII, in ORF, similar to human GGT
4-H12	0.6	28.4	71	2.5	10ug/ml	ACS1 promoter,
5-F6	1.4	29	69.6	2.4	>30ug/ml	peroxisome assembly protein, 3' end
6-B5	1.4	27.7	70.9	2.56	10ug/ml	YHL043w, small membrane protein
6-G9	1.7	28.3	70	2.47	>30ug/ml	YNR030w, similar to SMP3
8-E2	2	22.5	75.5	3.36	6ug/ml	TFCI, the 95kD chain of TFCIII
9-B5	2.2	24.5	73.3	3	8ug/ml	YBR176w
10-G10	4.4	23.4	72.2	3.08	6ug/ml	putative helicase
11-A11	3.9	30.4	65.7	2.16	8.5ug/ml	RAD23
11-B11	3.3	28.5	68.2	2.39	8.5ug/ml	Z48952
15-D5	1.2	30.8	68	2.21	4ug/ml	SLG1
21-D5	1.7	33.9	64.4	1.9	13ug/ml	X87331
25-H2	2.1	34.9	63	1.81	5ug/ml	sps2 homolog
31-G3	2.2	32.5	65.3	2.1	8.5ug/ml	SPE3
33-A3	4.1	32	63.9	2	5ug/ml	CWP2
33-H4	4	37.1	58.9	1.59	18ug/ml	ALG9
34-C2	3.2	31	65.8	2.12	6ug/ml	SLN1
35-A12	1.1	26.7	72.2	2.7	6ug/ml	YHL043w
35-B4	2	25.8	72.2	2.8	5ug/ml	X01736
35-F10	1.9	27.6	70.3	2.54	6ug/ml	IMP2
37-H5	1.3	33.4	63.3	1.9	5.5ug/ml	SLG1
38-A5	2.9	34.6	62.5	1.81	6ug/ml	Chr XV 800001-810
39-C11	1.5	36.2	62.3	1.72	4ug/ml	YCK2
44-B5	3.3	33.9	62.8	1.85	4ug/ml	TFC1
44-H11	3.1	34.1	62.8	1.84	15ug/ml	BUD4
46-E7	7.9	72.3	19.8	0.27	<3ug/ml	VAN1
46-C10	4.1	30.8	65.1	2.12	3ug/ml	U19103
Low	4.3	24.2	71.5	2.96	3ug/ml	SNF3
High	3.8	35.4	60.8	1.72	20ug/ml	SSK2

Figure 1

CFW/CGR Initial Screen

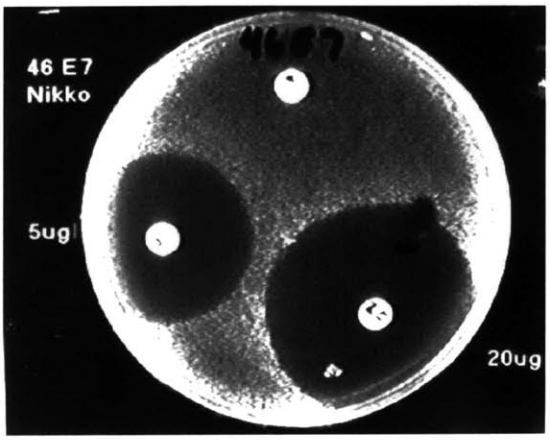


Putative positives were diluted and spot tested
on various concentrations of CFW and CGR

Figure 2

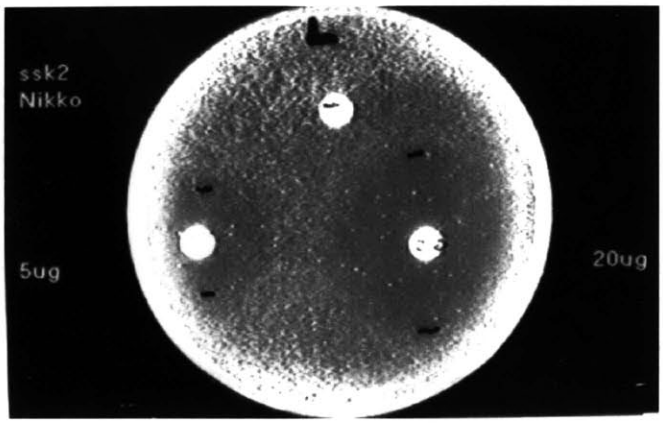
Agar Diffusion Assay for Drug Sensitivity

Nikkomycin Z



van1

(clear zones of inhibition)



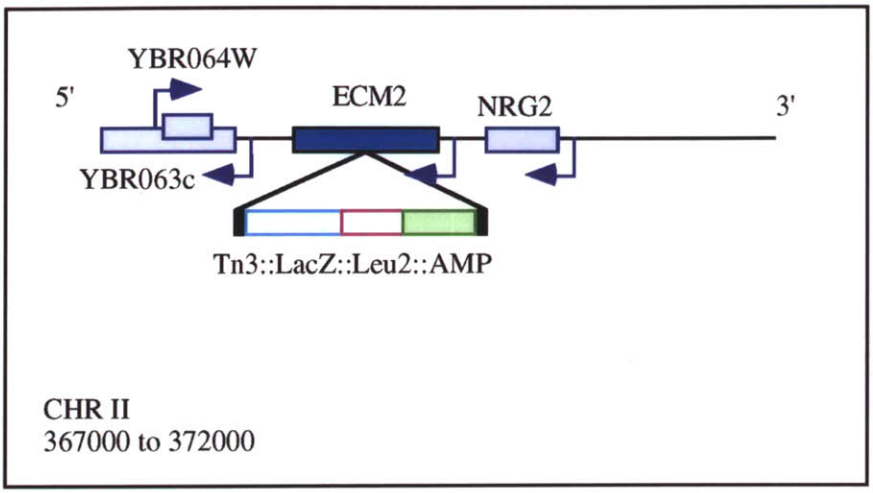
ssk2

(hazy zones of inhibition)

Three sterile 5mm paper disks were placed on medium embedded cells. One disk served as a no drug control, the second for a "low" amount of drug and the third for a "high" amount of drug. Cells were allowed to grow for 1 to 2 days and then scored by measuring the zones of growth inhibition.

Database Analysis of "Droopy Bud" Genes

ECM2



Function: Synthetic lethal with U2 snRNA; blocks pre-mRNA splicing *in vivo* and *in vitro*.

Synonyms: SLT11, YBR065c

Sequence Similarity: 27% homology to ribosomal protein L25 in central region (residues 151-299)

Glucose/Mannose Ratio: same as parent

CFW hypersensitivity/resistance: hypersensitive

CGR hypersensitivity/resistance: resistant?

Additional Information: Encodes a 364aa protein. Zymolyase, Hygromycin, Papulacandin, K1 Killer Toxin, and Caffeine hypersensitive (2). Null mutant is viable. Synthetically lethal in combination with *slt5/cdc40/slu4, slu7,* and *prp16* (3). N-terminal domain contains two putative zinc fingers (3). *slt1-1* blocks splicing prior to the first step, but has no apparent effect on spliceosome assembly (3). Tn3 mutant has "droopy bud" phenotype (2).

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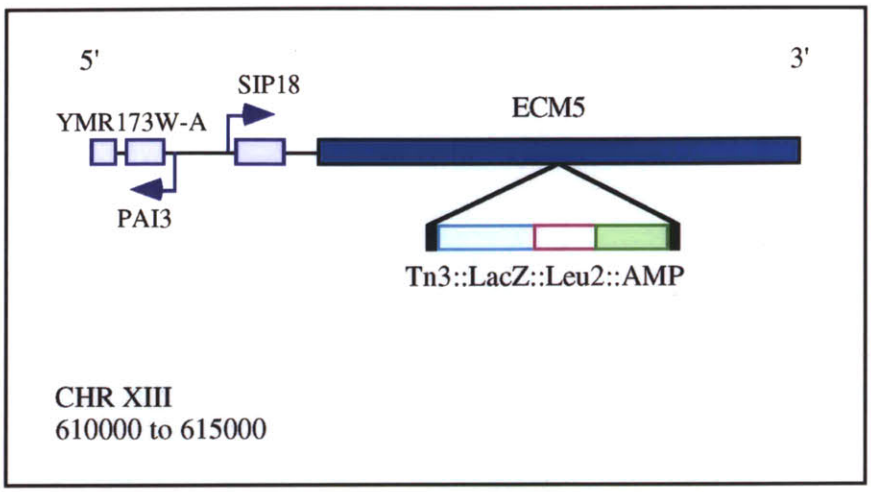
ECM5



Function: unknown

Synonyms: YMR176w, YM8010.06

Sequence Similarity: 27% homology with SW:X169_Human

Glucose/Mannose Ratio: Increased with respect to parent

CFW hypersensitivity/resistance: hypersensitive

CGR hypersensitivity/resistance: hypersensitive

Additional Information: Encodes a 1410aa protein. Null mutant is viable(1, 2). Increase in the amount of N-acetylglucosamine content (2). Hypersensitive to caffeine and Hygromycin B (2). Contains an ATP/GTP binding site (2).

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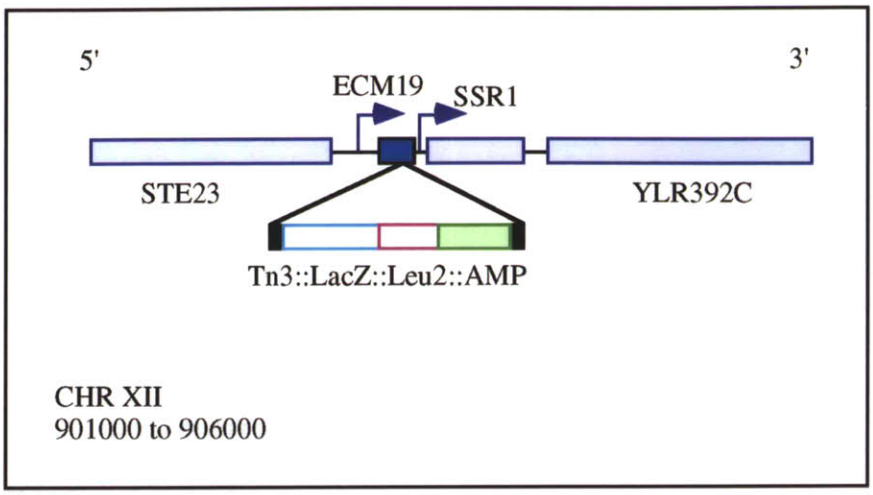
ECM19



Function: unknown

Synonyms: YLR390w

Sequence Similarity: none identified

Glucose/Mannose Ratio: increased with respect to parent

CFW hypersensitivity/resistance: hypersensitive

CGR hypersensitivity/resistance: hypersensitive

Additional Information: Encodes a 112aa protein. Null mutant is viable. Hypersensitive to Hygromycin B, K1 Killer Toxin, and Zymolyase (3). Contains two putative stress response elements in the promoter. Is present in about 14 copies per cell (2).

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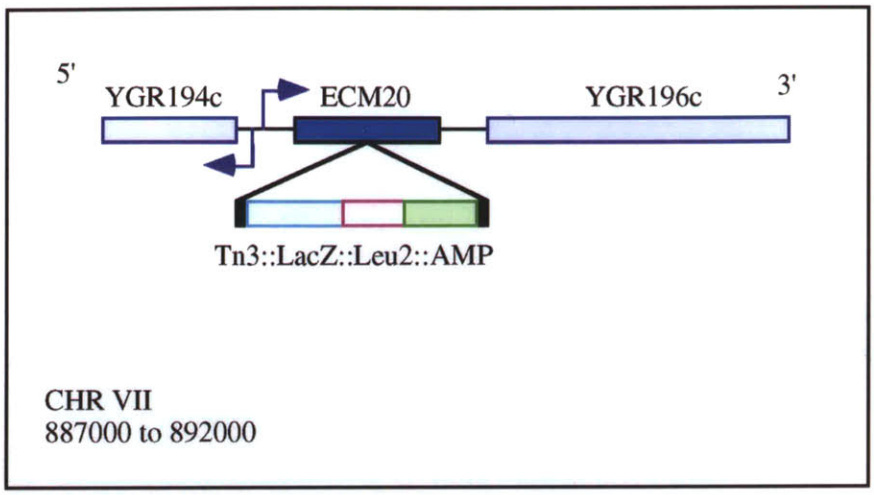
ECM20



Function: Required for normal 3' to 5' mRNA decay. Required for proper 3' end processing of 5.8S rRNA. Represses copy number of K1 killer toxin encoding M1 dsRNA.

Synonyms: SKI6, RRP41, YGR195w, G7587

Sequence Similarity: 25% homology to *E. coli* ribonuclease PH. Weak similarity to Mtr3p.

Glucose/Mannose Ratio: Increased with respect to mutant.

CFW hypersensitivity/resistance: hypersensitive

CGR hypersensitivity/resistance: hypersensitive

Additional Information: Encodes 245aa protein. Null mutant is lethal. Hypersensitive to K1 killer toxin, Hygromycin B, caffeine, Papulacandin (5).

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formation of 5.8S rRNA in *Saccharomyces cerevisiae*. *EMBO J* **17:** 1128-1140.

3. Edskes, H.K., Ohtake, Y., and Wickner, R.B. 1998. Mak21p of *Saccharomyces cerevisiae*, a homolog of human CAATT-binding protein, is essential for 60S ribosomal subunit biogenesis. *J. Biol. Chem.* **273:** 28912-28920.

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III. *Saccharomyces cerevisiae* Cell Surface Assembly Mutants With A "Droopy Bud" Phenotype

A. Introduction

The cell wall determines the ultimate shape and outer structure of the fungal cell. As a result, all the cell surface assembly mutants were examined for altered morphology. Of the mutants isolated through the transposon mutagenesis screen, 4 exhibited an additional "droopy bud" phenotype. These mutants are all 2-3 times larger than wildtype and have an elongated bud neck with a large bud "drooping" off the end. In addition, after staining the cells with Calcofluor White and observing them under fluorescent light, it is clear that the cells have an abnormally large amount of chitin in the bud neck region, and have an increased amount of delocalized chitin thoughout the cell wall. In an attempt to further characterize these mutants, deletions of the transposon mutagenized genes were structured using a PCR-mediated approach. The original transposon mutagenized strains and the deletion disrupted mutants were subjected to morphologic examination through the cell cycle, osmotic sensitivity assays, glycosylation assays, and chitin assays. In addition, three of the four mutants were tested for synthetic lethality in combination with *CHS3* (chapter IV this thesis).

B. Materials and Methods

1. Strains

Saccharomyces cerevisiae strains used in these studies have been previously described (chapter II this thesis) and are listed in Table 1.

2. Media and Growth Conditions.

Cells were grown at 30°C either on plates or in liquid media with aeration, unless otherwise noted. Four different media were used: YPD, SD, PSP2 and Nitrogen Starvation Media (NSM). YPD and SD have been described previously (this thesis chapter II). PSP2 is comprised of 6.7g yeast nitrogen base without amino acids, 1g yeast extract, 10g potassium acetate in 1 liter of 50m*M* potassium phthalate buffer (pH 5.0) (Kassir and Simchen, 1991). Potassium hydrogen phthalate was obtained from Sigma. NSM is comprised of 0.17% yeast-nitrogen base without amino acids and ammonium sulfate and 2% glucose (Segev and Botstein 1987). SD media were supplemented with the appropriate amino acids for strain growth.

3. Sequence Comparison

DNA sequence and protein homology searches were conducted through the Genbank Database at the National Center for Biotechnology Infomation (NCBI) (http://ww.ncbi.nlm.nih.gov), the *Saccharomyces* Genome Database (SGD) (http://genome-www.stanford.edu/Saccharomyces/), and the Yeast Proteome Database

(YPD) (http://quest7.proteome.com/YPDhome.html). Sequences obtained through Genbank, SGD and YPD were analyzed through the BLAST , DNASTAR, and GeneJockey programs.

4. Morphologic Examination

Parent and mutant strains were grown at 30°C in liquid medium. Exponentially growing cultures were resuspended in either NSM or PSP2 for growth arrest at 30°C. Cells were incubated in growth arrest media for 2-24 hours. Aliquots were removed and briefly sonicated to disrupt cell aggregates. Cells were observed under a light microscope to determine secession of growth. Cells were then shifted to either YPD or SD and 500µl aliquots were removed at various incubation periods to monitor synchronous growth through the cell cycle.

Each aliquot was stained with Calcofluor White by the addition of 5µl of a 10mg/ml in 50% ETOH solution of Calcofluor White. Stained cells, 5µl, were observed under bright and fluorescent light at 100X using a Zeiss Axioplan microscope.

5. PCR-Mediated Deletion

Deletions were created using a PCR-mediated protocol (Baudin *et.al.*, 1993, Wach *et. al.*, 1994 and Mishra *et.al.*, 1997). In brief, PCR primers were designed to amplify both 5' and 3' flanking sequence of the genes in question. In addition, the 3' left flank primer had the sequence at the 3'

end of *HIS3*. The 5' right flank primer had the sequence at the 5' end of *HIS3*. (Table 2, Figure 4). PCR reactions (100µl) contained PCRII buffer (Perkin Elmer), 2.5mM $MgCl_2$, 200nM each dNTP, 1mM each primer, 1µg genomic DNA from AWM3CΔ630 and 2.5 units Taq Polymerase. Cycling conditions were as follows: 1X 10 min at 94°C(30X 1min at 94°C, 1 min at 55°C, 1 min at 72°C) 1X 10 minutes at 72°C. The PCR product was purified in a 1% low-melt agarose gel. The band was cut out, melted in boiling water, and 5µl-10µl was used as primer DNA to fuse *HIS3* with the flanking gene sequence. The resulting PCR products were purified from low-melt agarose using Promega Qiagen gel extraction kit and transformed into yeast strains DB2, DB3, and DB4. The resulting transformants were checked on a Southern blot for the presence of *HIS3*. The resulting deletion mutants were tested for morphologic changes and chitin content.

6. Invertase Gel

The invertase gel protocol is described in Ballou, 1991. Briefly, 5ml of an overnight liquid YPD culture was washed twice in sterile PBS. The cells were resuspended in 5ml YEP with 0.05% glucose to induce invertase. Invertase is induced spontaneously when glucose in exhausted. Cells were grown at RT for 2-4 hours. Cells were placed on ice and washed with ice-cold 20mM sodium azide. Cells were then washed with 5ml ice-cold TBP buffer (5.52 diethylbarbituric acid, kindly provided by Susan Southard,

and 1g Tris base per liter). 1ml PMSF (0.174 phenylmethanesulfonyl fluoride in 10 ml ETOH) is added to 100ml TBP immediately before use). Cells were resuspended in 20µl cold TBP in a 1.5ml Eppendorf tube and placed on ice. Glass beads (0.45mm) were added to the meniscus. Cells were vortexed 5 times in 30 sec. pulses (fragile cells were vortexed twice). Cells were placed on ice between each pulses. 50µl TBP buffer with 15% glycerol and 0.01% bromphenol blue was added. Cells were centrifuged briefly at 14,000 rpm. Supernatant was placed in a separate tube. 5µl to 20µl was run on a native polyacrylamide gel. The remainder of the supernatant was frozen immediately.

The native gel was placed in 50ml of cold sucrose solution (0.1M pure sucrose in 0.1M sodium acetate, pH 5.1) for 10 minutes at 4^0C to allow the sucrose to diffuse into the gel. The gel was then transferred to fresh 37^0C sucrose solution and allowed to incubate at 37^0C for 10 minutes to allow invertase to hydrolyze the substrate. After rinsing twice with water, the gel was transferred to a Pyrex dish with 50ml fresh TTC solution (50mg 2,3,5-triphenyltetrazolium chloride in 50 ml NaOH) and heated on a hotplate in the hood until color developed. The gel was then rinsed thoroughly with water and fixed in 10% acetic acid. The gel was dried using a standard vacuum dryer.

7. Osmotic Sensitivity

Aliquots of exponentially growing cells (1ml) were resuspended in H_2O and H_2O containing 10% sorbitol. Cells were examined under the microscope at various intervals and 100µl aliquots were diluted and plated on solid YPD.

8. Chitin Analysis

colorimetric chitin analysis was carried out as described in Bulawa, (1992) and Wheat (1966). Briefly, exponentially growing cells were pelleted and resuspended in 1 ml hot 6% KOH. Cells were then incubated at 80°C for 90 minutes. Glacial acetic acid (0.1ml) was added and the cells centrifuged to collect all insoluble material. Pellets were washed twice with water and resuspended in 0.5ml 50mM sodium phosphate pH.6.3. 0.1U chitinase was added and the tubes were incubated at 37°C for 1 hour. Tubes were centrifuged at 14,000 rpm and the supernatant was removed to a separate tube. 400µl was used for the colorimetric Morgan-Elson reaction for GlcNAc determination.

9. Chitin Synthase III Assay

Bulawa, 1992 describes the chitin synthase III assay used. Briefly, 1 liter of exponentially growing cells at 30°C were pelletted, washed in buffer A (50mM Tris HCl, pH. 7.5, 1mM EDTA and 1mM DTT) and 0.5ml was removed for use. The remainder was frozen in a dry ice bath immediately. The 0.5ml pellet was resuspended in 2ml buffer A. Acid

washed 0.45mm glass beads were added to the meniscus. The samples were vortexed for 30 sec then placed on ice. This was repeated 4 times. The extract was transferred to a clean tube and the beads washed 4 times with buffer A. All washes were eventually combined with the initial extract. The extract was centrifuged in the cold for 3 minutes at 2,000 x g. The supernatant was removed. Cell walls in the pellet were washed again with buffer A. The total volume was increased to 20ml buffer A and the samples were centrifuged at 4^0C in a Beckman 50Ti rotor at 35,000rpm for 45 minutes. The pellets were resuspended in buffer B (identical to buffer A without DTT). Membranes (20µl) were incubated with 1mM UDP-[U-^{14}C] *N*-acetylglucosamine, 40mM *N*-acetylglucosamine, 50mM Tris-HCl (pH. 7.5), and 5mM $MgCl_2$ at 25^0C. The reaction was stopped by the addition of cold 10% TCA. The reaction was filtered onto a glass disc and counted. All results were recorded as comparisons to wild type.

10. Sugar Composition

Carbohydrate analysis was conducted as described previously (this thesis chapter II). Amounts of mannose, glucose, and GlcNAc were assayed and data is presented as amounts relative to the parent strain.

C. Results

1. Sequence Analysis

The results of the sequence analysis and database searches are summarized in the Chapter II Appendix section 4 "Droopy Bud Mutants". Briefly, the sequences are not in any way homologous to each other, and they contain no similar structures. *ECM2* has also been identified as *SLT11*, a temperature sensitive gene which is a synthetic lethal with U2 snRNA. It has a 27% identity to ribosomal protein L25, contains 2 putative zinc fingers in the N-terminal domain and blocks pre-mRNA splicing *in vivo* and *in vitro*. *ECM5* has no known function. It has a 27% identity to *SW:X169_Human* and contains an ATP/GTP binding site. *ECM19* seems to be regulated by the stress response. It contains two putative stress response elements in the promoter and is thought to have about 14 mRNA copies per cell. *ECM20* has also be identified as *RRP41*, and *SKI6* a ribosomal RNA processing homologue of RNAse PH which acts as a 3'-5' processive exoribonuclease which generates 5' diphosphates. A null mutation is lethal. When functioning, it represses the expression of M1 dsRNA which encodes Killer Toxin. As a result the mutant shows a superkiller phenotype.

2. Morphologic Examination

Figures 1 and 2 show the phenotypes of DB2, DB3, DB4 and AWM3CΔ630 through the cell cycle. As can be seen, DB2, DB3 and DB4 are larger in size than the parent strain, chitin is delocalized throughout the

mother cell and there is an increase in chitin at the mother-daughter junction. In addition, the bud droops dramatically.

3. PCR-mediated Deletion

The structures of the deletion mutants can be seen in Figure 4. Deletion of *ECM5* is lethal. Figure 3 shows that deletions of *ECM19* (DB5) and *ECM20* (DB6) slightly enhance the altered morphology of DB3 and DB4.

4. Invertase Gel

Figure 5 shows that DB2, DB3 and DB4 are not defective in glycoslyation as measured by the size of invertase. Invertase is a secreted protein with 14 potential glycoslyation sites. The rate at which invertase migrates in a native gel depends on the number and size of N-linked oligosaccharide chains attached. Any defect in glycoslyation will appear as a difference in invertase migration. As Figure 5 shows, DB2, DB3 and DB4 migrate identically with the parent wild-type strain AWM3CΔ630 showing that the mutants are not defective in glycoslyation.

5. Osmotic Sensitivity

As seen in Table 1, there is no change in the viability or morphology of the mutants after being placed in water for up to 2 hours.

6. Chitin Analysis

Chitin analysis was performed on DB2, DB3 and DB4. As can be seen in Table1 there is a 10-40% increase in the amount of chitin produced in these strains. This was expected given the carbohydrate levels in the cells.

7. Chitin Synthase Analysis

There appears to be a slight increase in the amount of chitin synthase activity in DB2, DB3 and DB4 relative to the parent strain (data not shown). However, the results from this series of experiments were variable and further study is needed.

8. Sugar Composition

Table 1 shows the sugar composition of the mutants. As can be seen, there is an increase in the mannose/glucose ratios and an increase in the levels of GlcNAc. This result agrees with the finding that there is an increased amount of chitin in the cell.

D. Discussion

The four "droopy bud" mutants were interesting because they showed similar morphology, yet the genes involved had no sequence similarity and, when I began the research, none had any previously described function. As can be seen from the fluorescent staining, there appears to be an increase in delocalization of chitin throughout the cell wall. Most striking is the appearance of DB3 which resembles DAPI staining of a *Drosopholia* embryo showing gradients of developmental signals. In addition, all four strains (DB1, DB2, DB3, DB4) show varying

degrees of increased chitin localization at the mother-bud junction. In an effort to understand this altered chitin localization, I began experiments which analyzed the components of the cell walls of the strains. DB1 was the strain I began to analyze initially. Unfortunately, it grew very poorly, and repeated attempts to revive the culture from frozen stocks failed. As a result, I attempted to construct a deletion strain directly with little success.

All three mutants have an increase in the amount of GlcNAc present in the cell wall, along with an increase in the ratio of mannose to glucose. They all have an increase in the amount of chitin present in the cell wall and the data suggests that there is a slight increase in the activity of *CHSIII*. The question remains, though, what does a gene involved in pre-mRNA splicing (DB1), a gene which when absent confers a superkiller phenotype and encodes a ribosomal RNA processing protein (DB4), a gene which appears to be regulated by the stress response and has about 14 copies of mRNA per cell, (DB3)(Appendix this section), and a gene of unknown function have to do with each other and the cell wall? It would be easy to look at these mutants and say that they do not have any direct interaction with the cell wall, that any phenotype is secondary to the major activity of these genes. It seems possible that at least one gene, *ECM19*, functions to help maintain the integrity of the cell wall throughout the cell cycle and interacts directly with chitin synthase III. *ECM19* (DB3)

is synthetically lethal in combination with *CHS3* (this thesis Chapter IV). It is also appears to be regulated by the stress response heat shock proteins. (Moskvina, E. *et.al.,* 1998) DB3 could serve to up regulate *CHS3* under conditions of environmental stress. A simple experiment to test this hypothesis would be to measure levels of *CHS3* activity in the mutant and parent strains after subjecting the cells to shock.

DB4 (*ECM20)* is hypersensitive to Papulacandin B, an inhibitor of glucan synthase. It is hypersensitive to K1 Killer Toxin and it decreases the amount of M1, Killer Toxin encoding, double-stranded RNA (Masison, D.C. 1995). This ribosomal RNA processing protein is part of the 3'-5' exosome, and though it specifically binds Killer Toxin RNA, it probably does not specifically bind any of the glucan synthases RNAs. However, a northern blot of a wild type strain and DB4 with RNA samples taken over time, and probed with glucan synthase sequence, might serve to shed light on the question.

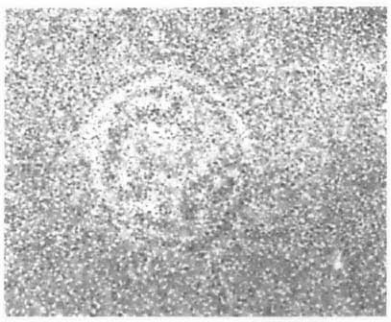
There is no evidence for the interaction of *ECM5* (DB2) and any of the chitin synthases or the glucan synthases. To attempt to identify a physical interaction between *ECM20* and the glucan or chitin synthases, I propose a 2 hybrid screen with *ECM20* as the bait. This experiment would allow for the isolation of genes directly involved with *ECM20* and involves less strain construction than a synthetic lethal screen for each of the chitin synthases and the glucan synthases.

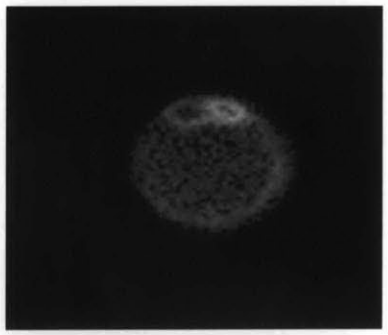
It is clear that mutations in *ECM5, ECM19* , and *ECM20* profoundly effect the *Saccharomyces cerevisiae* cell surface organization. Further study is needed to specifically understand their role in maintaining the cell wall throughout the cell cycle and during environmental stress.

E. Figures

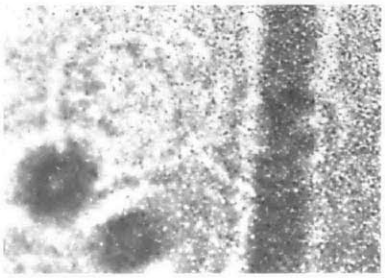
Figure 1

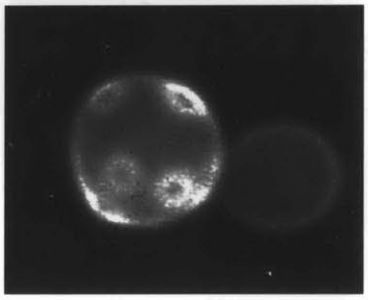
Un-Budded Cells



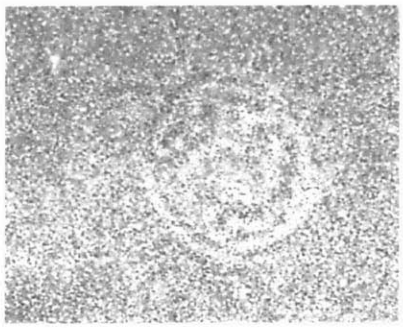


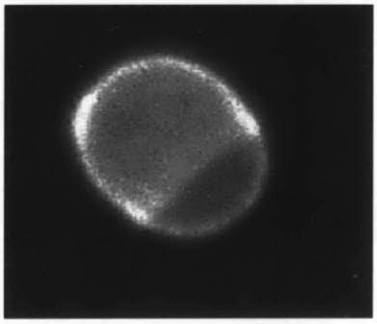
AWM630



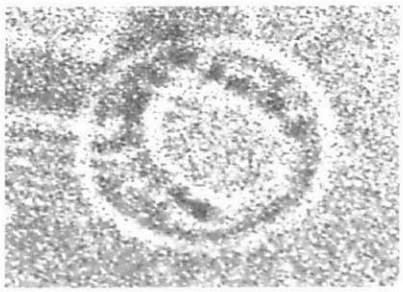


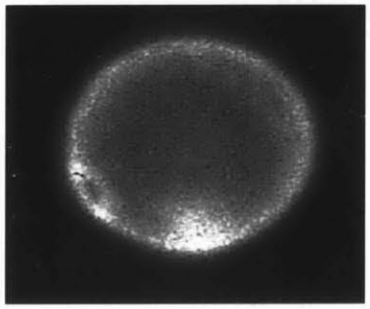
DB2





DB3

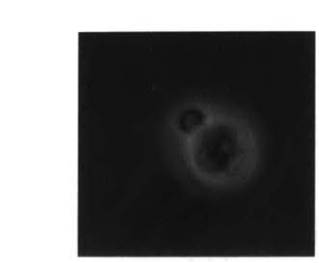




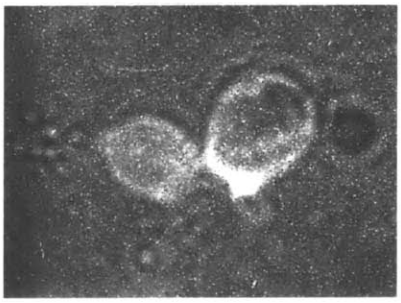
DB4

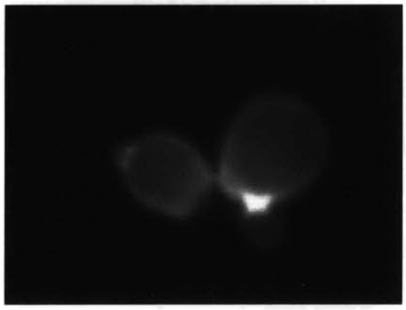
Figure 2

Budding Cells

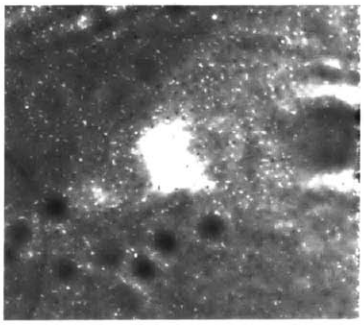


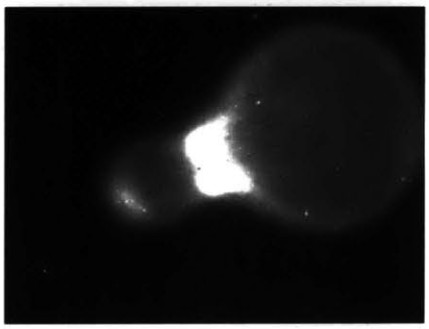
AWM630



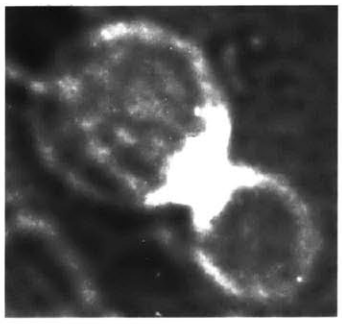


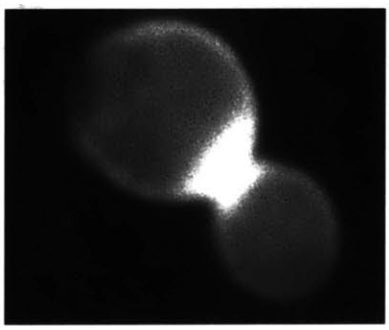
DB2





DB3

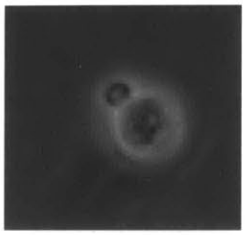


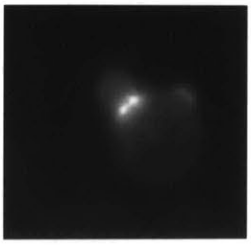


DB4

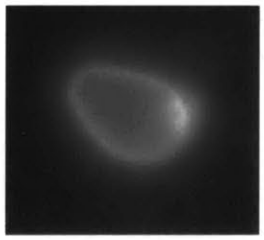
Figure 3

Deletion Mutants

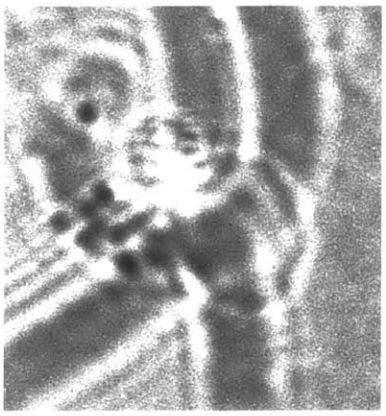


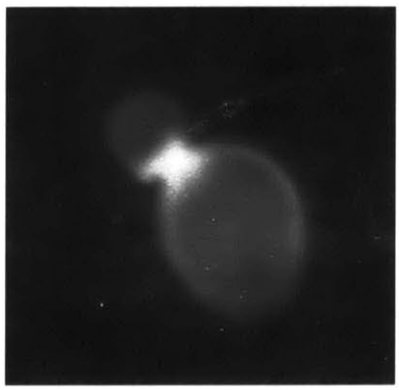


AWM630



DB5





DB6

Figure 4

PCR Mutagenesis

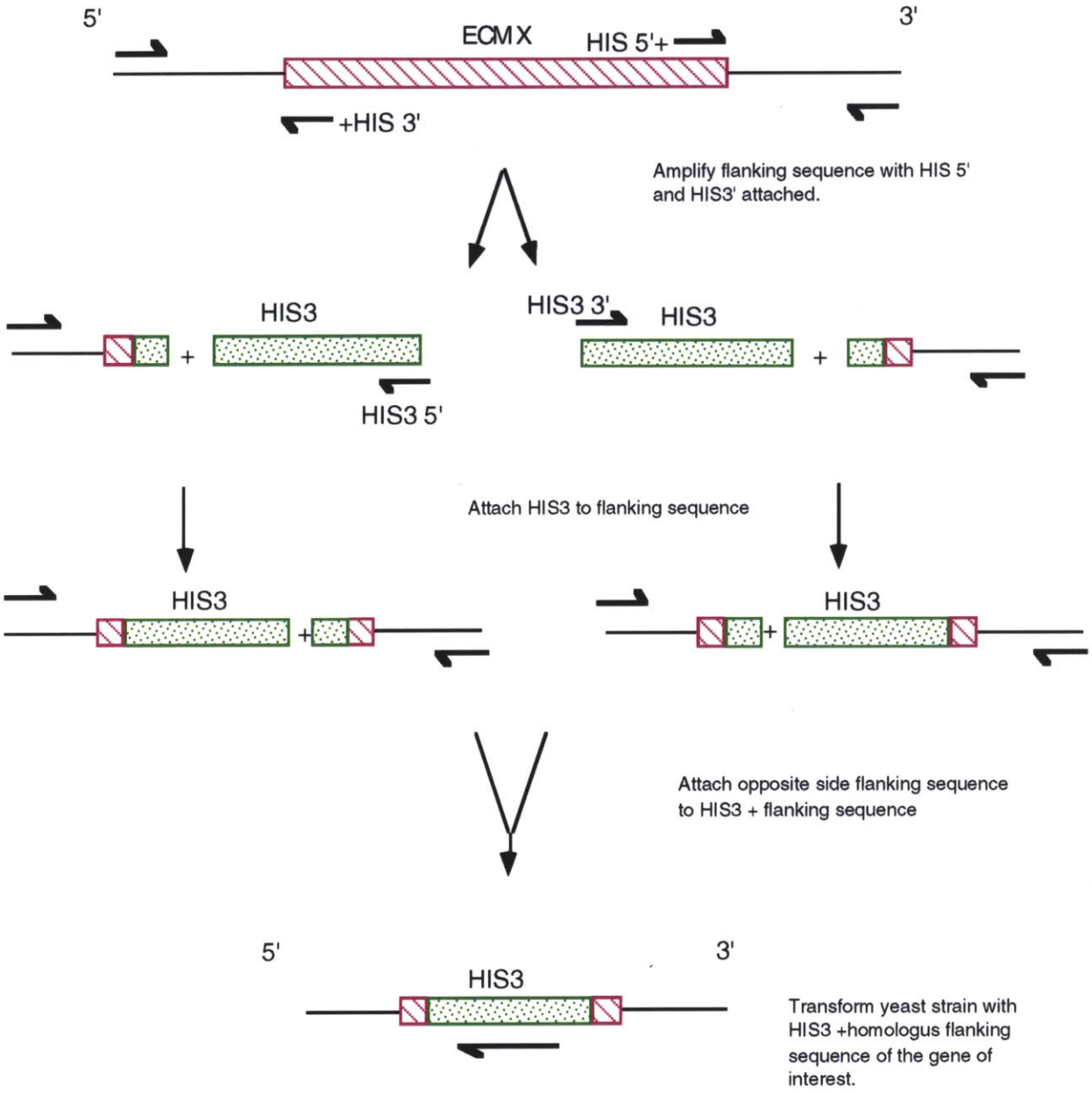
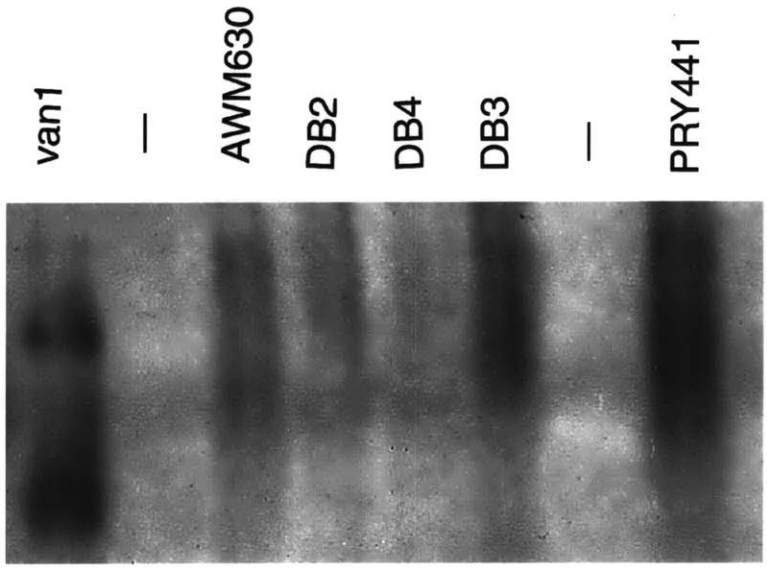
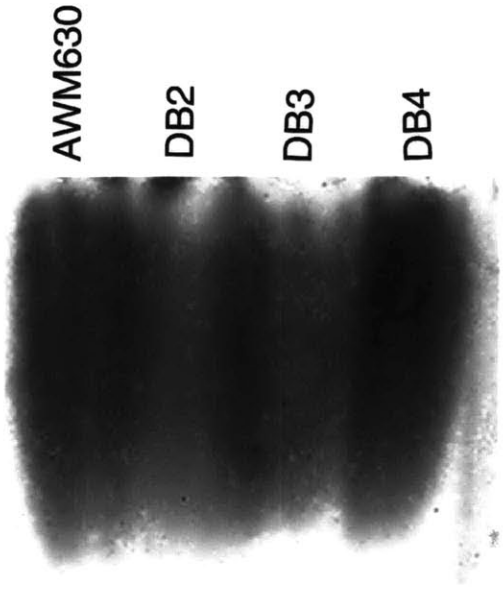


Figure 5

Invertase Gels





Invertase is a highly glyscolyated, secreted protein. Mutants with glyscolyation defects show altered invertase glyscolyation. The rate at which invertase runs on a native gel depends on the number and size of N-linked oligosaccharides attached.

F. Tables

Table 1

Strains Used

Name	Genotype	Tn3 mutagenized gene
AWM3CΔ630	*Mat* **a**, *ura3-639, leu2-3, 2-112, his3-11, 3-15, cir*^{0}	none, parent strain
DB2	*Mat* **a**, *ura3-639, leu2-3, 2-112, his3-11, 3-15, cir*^{0}, *Tn3::LEU2::lacZ::URA3::ecm5*	*ECM5*
DB3	*Mat* **a**, *ura3-639, leu2-3, 2-112, his3-11, 3-15, cir*^{0}, *Tn3::LEU2::LacZ::URA3::ecm19*	*ECM19*
DB4	*Mat* **a**, *ura3-639, leu2-3, 2-112, his3-11, 3-15, cir*^{0}, *Tn3::Leu2::lacZ::URA3::ecm20*	*ECM20*
DB5	*Mat* **a**, *ura3-639, leu2-3, 2-112, his3-11, 3-15, cir*^{0}, *HIS3::ecm5*	*ECM5*
DB6	*Mat* **a**, *ura3-639, leu2-3, 2-112, his3-11, 3-15, cir*^{0}, *HIS3::ecm19*	*ECM19*

Table 2

PCR primers

Gene	Location	Sequence
ECM19	left flank 5'	GAGACTGATAGGTGAGAGGTGAACC
	left flank 3'	CGTGTCATTCTGAACGACATCGCTTTCCCCTAGTTTCTGCGG
	right flank 5'	CTAGAGGAGGCCAAGAGGCACCACATGCATTAGGACCCA
	right flank 3'	GGCCTTGCTGGATGAAGAAGCGG
ECM20	left flank 5'	GGACAGCAGAAGCTCAGA
	left flank 3'	CGTGTCATTCTGAACGACATCATCCTGCTGTAGAA
	right flank 5'	CTAGAGGAGGCCAAGAGGTTTCATAGAATGCTGG
	right flank 3'	GGCCTTACGATCAGGGTTCG
ECM5	left flank5'	GTCCGAAGGGGGAATGAAGATGGGCC
	left flank 3'	CGTGTCATTCTGAACGACCTCCGAAGCGCTACACCTCC
	right flank 5'	CTAGAGGAGGCCAAGAGGGTTGGCAGTAGTTACCT
	right flank 3'	GCTCGGTCTCTGAATCACG
HIS3	3'	CGTGTCATTCTGAACGA
	5'	CTAGAGGAGGCCAAGAG

Table 3

"Droopy Bud" Mutant Phenotype

Name	Os. Sen.[1]	T. Sen[2]	GlcNAc	Man/Glu	Chitin[3]	Δ Viability[5]	Glyc[6]
DB1[6]	NT[7]	NT[7]	same	same	NT[7]	NT[7,6]	NT[7]
DB2	no	no	inc	inc	10%-25%	lethal	same
DB3	no	no	inc	inc	40%	not lethal	same
DB4	no	no	inc	inc	15%-30%	not lethal	same

[1] Osmotic sensitivity determined by placing cells in water for 2 hours, removing aliquots and observing under the light micr and by plating dilutions to determine colony forming units.

[2] Temperature sensitivity determined by placing cells at 26°C, 30°C, 32°C, and 37°C. Growth was monitored using microscopy an checking for colony forming units.

[3] Chitin content is recorded as percentage increase relative to parent chitin levels.

[4] Δ Viability = Deletion anaylsis

[5] Glycosylation analysis determined by running invertase gels and monitoring enzyme migration of mutants relative to pare enzyme migration

[6] CSA1 was an extremely fragile slow growing mutant. It eventually did not survive as a frozen stock.

[7] NT = not tested

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IV. GENES WHICH GENETICALLY INTERACT WITH *CHS3**

*Work included in this chapter was done in close collaboration with Barbara C. Osmond and Paul D. Awald. I would like to thank Barbara C. Osmond for conducting a majority of the work reported in this chapter and also for writing a majority of this chapter for publication.

A. INTRODUCTION

As previously noted, there are three chitin synthase genes in *Saccharomyces*, chitin synthase I (*CHS1*), chitin synthase II (*CHS2*), and chitin synthase III (*CHS3*). Chitin synthase I is required for normal budding under the acidic conditions following mother-daughter cell separation. (Cabib *et. al., 1992,* Bulawa, 1993). However, Δ*chs1* mutants show no obvious phenotypic differences with respect to chitin localization, mating or budding, but they lack much *in vitro* chitin synthase activity. *CHS1* is 10 to 20 times more active in extracts than *CHS2* or *CHS3.* It is thought that *CHS1* deposits "repair" chitin in the neck between the mother and the daughter cell (Cabib *et.al., 1992*). Chitin Synthase II (*CHS2*) is required to maintain proper cell morphology and normal cell separation, including septum formation (Sliverman *et.al.,* 1988; Bulawa, 1993). Chitin synthase III *(CHS3)* produces greater than 90% of all the chitin in the cell and its expression is governed by *CHS3, CHS4 (CSD4, CAL2, SKT5),* and *CAL3* (Bulawa, 1993 Bulawa, *et.al.,* 1986). It is responsible for the synthesis of chitin in the ring and the lateral wall. *CHS3* also regulates the synthesis of nascent strands of chitin which form chitosan and encodes the catalytic subunit of the major chitin synthase (Bulawa, 19992; Roncero *et.al.,* 1998; Pammer *et.al.,* 1992; Takita and Castilho-Valavicius, 1993). In

addition, it is the chitin produced by *CHS3* which is linked to β-(1,3)-glucan.

Chitin synthase III is regulated by chitin synthase IV (*CHS4* (*CSD4, CAL2, SKT2*)) and chitin synthase V (*CHS5* (*CAL3*)) both *in vivo* and *in vitro*. It is thought that *CHS4* acts to both up and down regulate *CHS3* and chs3p containing complexes (Bulawa, 1993). Chitin Synthase V (*CHS5*) may synthesize a protein which functions similarly to *CHS4* or it may be involved in localization of chs3p since it has been shown that Δ*CHS5* results in a loss of chitin localization at the bud neck and bud site (Santos and Snyder, 1997). Interestingly, Δ*chs1*/Δ*chs2* and Δ*chs1*/Δ*chs3* are viable, Δ*chs1*/Δ*chs2*/Δ*chs3* is not, showing that chitin synthesis is essential (Bulawa and Osmond, 1990, Shaw *et. al.*, 1991, Bulawa, 1992, Bulawa, 1991).

While an increasing number of genes involved in cell surface assembly are being identified (Ram *et. al.*,; Lussier *et. al.*, 1997), the identification of genes involved in the regulation of chitin is far from complete. In order to identify additional genes directly or indirectly involved in chitin synthesis regulation, we conducted a synthetic lethal screen on UV mutagenized cells and four previously identified cell surface assembly mutants. A synthetic lethal screen has the primary advantage of allowing for the identification of genes which are essential for viability in

combination with a specific gene or function. As a result, it is possible to characterize functionally related proteins, interacting proteins, regulatory proteins and proteins which belong to the same complex (Phizicky and Fields, 1995; Cid *et. al.*, 1995).

B. MATERIALS AND METHODS

1. Yeast Strains Media and Plasmids

Yeast strains used in this work are listed in Table 1. Yeast were grown in either rich medium (YPD) or in synthetic minimal (SD) which have been previously described (Sherman et al., 1986). Other media were SD^+ (SD plus adenine, histidine, uracil, leucine, lysine and tryptophan), SD^+ -ura (as SD^+ but lacking uracil), SD^+ -trp (as SD^+ but lacking tryptophan) and SD^+ -leu -trp (as SD^+ but lacking both leucine and tryptophan). Calcofluor white containing medium was made as previously described (Bulawa 1992). SD^+ medium was used for all drug tests with the exception of Nikkomycin Z. For Nikkomycin Z drug tests SDA^+ medium, which contains allantoin (1 mg/ml final concentration) as the nitrogen source, was used (Island *et al.*, 1987). Solid media contained 2% agar (Difco Laboratories), unless otherwise noted. Strains were grown at 26°C. and 30°C

2. Strain Construction

Standard procedures of yeast genetics were used, (Sherman *et al.*, 1986). Yeast transformations were conducted using the lithium acetate method (Soni *et al.*, 1993). Standard methods were used for the construction of plasmids. *Escherichia coli* strain DH5α was used for transformation and plasmid construction. *E. coli* were transformed using the procedure of Inoue *et al.*, (1990).

C. PLASMIDS

pBK101: The 3.7kb *Bam*HI/*Nhe*I fragment of pDK255 (Koshland *et al.*, 1985) containing *ADE3*, was cloned into the *Bam*HI/*Spe*I sites in the multiple cloning site (MCS) of the *TRP1* marked *CEN*6 vector pRS314 (Sikorski and Hieter, 1989) to make pBK101.

pBK102: pCSD2-15, (5.5kb *Cla*I-*Bam*HI fragment of pCSD2-3 cloned into pSK [Stratagene] made by C. Bulawa) was cut with *Bam*HI and *Sal*I. The 5.5kb band containing *CHS3* (*CSD2*) was gel purified and ligated to *Bam*HI/*Sal*I digested pBK101 to make pBK102.

pCSD2-3: Bulawa subcloned the 5.4 kb *Cla*I-*Bam*HI fragment of pCSD2 (Bulawa, 1992) into the same sites of the *CEN6/ARSH4* vector pRS316 (Sikorski and Hieter, 1989.)

pRS316: *URA3* marked *CEN6/ARSH4* vector described elsewhere (Sikorski and Hieter 1989).

p12a-1: *CEN4 URA3* library plasmid carrying complementing insert, *SRV2*

p13d-3: *CEN4 URA3* library plasmid carrying complementing insert, *FKS1*

p13a-1: *CEN4 URA3* library plasmid carrying complementing insert, *ANP1*

D. Yeast Genomic Library

The *CEN4 URA3* marked yeast genomic library was a generous gift from the Young Laboratory (Whitehead Institute, Cambridge, MA) and is described elsewhere (Thompson *et al.*, 1993).

E. Synthetic Lethal Screens

1. UV Mutagenesis

Strain PRY487 was grown on SD^+ -leu -trp. Individual colonies were suspended in 2ml of SD^+ -leu -trp and grown for 90 minutes at 26°C. A 1ml aliquot of each suspension was sonicated briefly to disperse clumps. Cell counts were done using a hemocytometer. Suspensions were diluted in H_2O and plated at 2 x 10^3 cells/plate on YPD. These cells (total of 1 x 10^5 cells) were mutagenized with UV irradiation to a viability of 6% (60 second exposure, 40 cm from source, lamp output 10ergs/mm^2), lids were replaced and incubated at 26°C in the dark.

2. Viable Counts

Suspensions were diluted with H_2O and plated for singles on YPD. After 3-5 days incubation, the number of colonies was determined to assess viability of unmutagenized cells. The ratio of the number of colonies on mutagenized plates to the number expected was used to determine percent viability of mutagenized cells.

3. Transposon Mutagenesis

Haploid strain AWM3CΔ630 was mutagenized using transposon Tn3::*LEU2*::*lacZ* according to the procedure outlined in Burns *et.al.*,(1994). Haploid strain AWM3CΔ630 was also mutagenized using PCR mutagenesis as described previously (Chapter III this thesis). Putative positives were screened for resistance or hypersensitivity to calcofluor white and congo red (Lussier, *et.al.*, 1997). Of the cell surface assembly mutants identified, 4 exhibited a "droopy bud" phenotype (DB1, DB2, DB3, DB4/ DB6).

4. Mating

Haploid strains DB2, DB3, DB4/DB6 were mated with PRY487 to test for synthetic lethality in combination with *CHS3*. Diploids were selected by plating on SD -his -ura at 30°C, sporulated on SPO plates at 26°C and tetrad analysis performed. Synthetic lethality was determined by identifying a "missing class" of spores. Results are summarized in Table 3.

5. Screening UV Mutagenized Cells for Synthetic Lethals

After 5 days incubation, colonies on mutagenized plates that appeared red without white sectors (Sect$^-$) were streaked for singles on YPD at 26 °C. Individual Sect$^-$ colonies were restreaked at least twice to confirm their non-sectoring phenotype.

6. Characterization of Putative UV Mutagenized Synthetic Lethal Mutants

Standard genetic procedures were used to determine the recessiveness or dominance of the mutants. Sect$^-$ mutants were mated to PRY398 and the resulting diploids were tested for their ability to sector by streaking on YPD. Tetrad analysis was performed on diploids whose sectoring phenotype was recessive to confirm that both Sect$^-$ and Sect$^+$ were recoverable. This also served to backcross the mutagenized strains.

Other studies that have used the *ade2 ade3* color colony sectoring assay to identify synthetic lethal mutants have found that a percentage of their mutants were Sect- because the plasmid carrying *ADE3* had integrated into the chromosome or the chromosomal *ade3* had undergone gene conversion or reversion to *ADE3* (Bender and Pringle, 1991). In order to rule out these false positives, all putative synthetic lethal mutant strains were mated to an *ade2 ade3* tester strain (PRY398) and the resulting diploids were tested for their ability to sector.

In addition, putative synthetic lethals were transformed with pRS316 and with the *CEN* plasmid pCSD2-3, which carries a wildtype copy

of *CHS3,* to verify the requirement for the plasmid borne *CHS3* for viability.

7. Agar Diffusion Assay for Drug Sensitivity

Agar diffusion assays were used to test synthetic lethal mutants for sensitivity to the following drugs: Hygromycin B, Amphotericin B, Nikkomycin Z, Tunicamycin, the echinocandin L-733,560, Sodium Orthovanadate, FK-506, Killer Toxin, Papulacandin, and Caffeine. The procedure used was a modified version of that described by Island *et al.* (1987). Briefly, strains were grown to mid log-phase at 26^0 C in SD^+ or SDA^+. Cells were washed and resuspended in sterile water to an OD_{600} of 0.10. Cell suspensions (780 µl) were added to 7.0 ml molten (43^0 C) SD medium containing 1% agar and overlaid on a standard SD^+ plate (2% agar). Three sterile 5mm paper disks were placed on the medium embedded cells. One disk served as a no drug control, the second for a "low" amount of drug and the third for a "high" amount of drug. In each case, 10µl of the appropriate drug dilution was applied to the disk. Cells were allowed to grow for 1 to 2 days (26^0C or 30^0C) and then scored by measuring the zones of growth inhibition. Results are summarized in Table 2.

Hygromycin B, Amphotericin B, Nikkomycin Z and Tunicamycin were obtained from Calbiochem. L-733,560, an echinocandin (Douglas *et al.* 1994, El-Sherbeini and Clemas, 1995), was the generous gift of Myra Kurtz

(Merck Research Laboratories, Rahway, NJ). Sodium Orthovanadate and Congo Red, Calcofluor White were from Sigma Chemicals. Papulacandin B was a generous gift from Howard Bussey (McGill University, Canada). K1 Killer Toxin was a generous gift from Howard Bussey (McGill University, Canada) Hygromycin B, Nikkomycin Z and Sodium Orthovanadate were dissolved in sterile water. Amphotericin B, L-733,560 and Tunicamycin were dissolved in 10% (v/v) DMSO. FK-506 was dissolved in 50% (v/v) methanol/DMSO. Congo Red and Calcofluor White were dissolved in 50% ethanol.

Strains were tested for calcofluor white and congo red sensitivity by streaking for singles on YPD plates containing 1, 5, 10, 20, 200, 500 and 700 μg/ml calcofluor white or congo red. In addition, cells were spot tested in varying dilutions on the aforementioned plates to obtain specific resistance or hypersensitivity values without titrating out the drug.

8. Cloning of the gene complementing the Sect- phenotype

We utilized the drug sensitivity of the synthetic lethal strains to facilitate the cloning of the genes which, when UV mutagenized, are synthetically lethal with a *CHS3* deletion. Synthetic lethal strains hypersensitive to Nikkomycin Z were transformed with the *CEN4 URA3* library and plated on SD^+ -ura -trp at a density of 1 x 10^3 cells/plate. After

3 to 4 days, transformants were replica plated to SDA$^+$ -ura -trp, plus 15μg/ml Nikkomycin Z. After 1 to 2 days incubation at 26^0 C, any transformants that had grown on the Nikkomycin Z plate were streaked for singles on YPD, and YPD plus 500 μg/ml Calcofluor White. Single colonies were re-tested for the presence of the plasmid(s) and the ability to grow on plates with 15 μg/ml Nikkomycin Z. Transformants that were able to sector on YPD and resistant to Calcofluor White and Nikkomycin Z underwent plasmid rescue (Hoffman and Winston, 1987).

Synthetic lethal strains that were sensitive to Calcofluor White were transformed with the *CEN4 URA3* library and plated on SD$^+$ -ura plates as described above. The SD$^+$ -ura plates contain tryptophan so there is no auxotrophic requirement for maintaining the *TRP1* marked plasmid carrying the *CHS3* gene. After 3 to 5 days, colonies were replica plated onto SD$^+$ -ura plus 700μg/ml calcofluor white. Calcofluor resistant colonies from library transformed strains were restreaked on YPD, YPD plus 700 μg/ml calcofluor, SD$^+$ -ura, SD$^+$ -trp and SD$^+$ plus 1 mg/ml 5-fluoroorotic acid (5-FOA), (Boeke, 1987).

Complementing library plasmid DNA was prepared from the yeast strain by the method of Hoffman and Winston (1987).

F. Sequencing

Double stranded DNA sequencing was carried out by the chain termination method (Sanger) using a cycle sequencing kit (Epicentre Technologies, Madison, WI) by extension of T3 and T7 promoter sequences present in the plasmid to obtain the 5' and 3' sequence of the complementing insert. Comparison of the deduced amino acid sequences to sequences present in GenBank and EMBL databases was carried out using BLAST (National Center for Biotechnology Information).

G. Cell Wall Assays

Cell wall assays (labeling and fractionation of cell wall polysaccharides) were done using a modified version of the procedure described by Castro *et al.*, (1995). Briefly, cells were grown, shifted to fresh medium at a low inocculum and grown for 2-3 hours. To 3ml of the liquid culture, ^{14}C-U-Glucose at 1mCi/ml was added and the cells were allowed to undergo three doublings. While still in log phase, cells were pelleted, and washed with water. Carrier cells, 150µl of packed unlabeled wild-type cells, were added to the washed labeled cells and the cells were then broken by "beadbeating" in water for 3 cycles of 4 minutes each with 5 minutes of cooling. Following centrifugation for 5 minutes at 3000 rpm, the pellet containing cell walls was washed 3 times with 5% NaCl and then 3 times with 1mM EDTA to yield a preparation of purified cell walls.

Cell walls were extracted 4 times with 6% NaOH for 45 minutes at 80^{0}C to yield two fractions, the pellet, or alkali insoluble fraction, and the supernatant, or alkali soluble fraction. The alkali insoluble fraction was washed until a neutral pH was attained, resuspended in 500 µl of 1mM EDTA, and an aliquot counted. This fraction consists of chitin and alkali-insoluble glucan. The alkali-soluble fraction was divided into two aliquots. One aliquot of the alkali soluble fraction was treated with the Fehling reagent , as previously described by Algranati *et al.* (1966), in order to precipitate the cell wall polysaccharide mannan. In the second aliquot, alkali-soluble β-glucan and mannan were coprecipitated by adding two volumes of ETOH. The difference in radioactivity between the two pellets, the mannan precipitated with the Fehling reagent and the mannan precipitated with ethanol, represents alkali soluble β-glucan. All determinations were carried out in triplicate.

H. β-Glucan Synthase Assays

Enzyme preparation and assays were done as previously described with minor modifications, (Castro *et al.*,1995). Briefly, early logarithmic cells were resuspended in 1mM EDTA (pH8) and lysed in a BeadBeater. The crude lysate was centrifuged at low speed to remove unbroken cells and cell wall debris. Following a high speed spin, washed pellets were resuspended in buffer plus glycerol and stored at -20^{0}C. β-1,3 Glucan

synthase reactions were done as previously described, (Castro *et al.*, 1995). The amount of [14C]-glucose incorporated into acid insoluble glucan was determined using a Millipore filter method previously described by Awald (thesis) (originally by Gooday and DE Rousset-Hall (1975). The amount of [14C]-glucose incorporated into insoluble glucan trapped on the filter was determined by liquid scintillation counting.

I. Results

We conducted two synthetic lethal screens to identify genes which are essential in combination with *CHS3*. In the first screen, an *ade2 ade3* strain containing both a deletion of *CHS3* and a plasmid with a wild-type copy of *CHS3* and *ADE2*, were mutagenized by UV irradiation and tested for ability to sector. Following mutagenesis, 23 non-sectoring colonies were recovered. These 23 putative synthetic lethal strains were further characterized. In the second screen, 3 transposon mutagenized strains were mated with a $\Delta chs3$ strain, sporulated and analyzed for missing spore classes.

1. Characterization of Putative Synthetic Lethal Strains

All 23 putative UV mutagenized synthetic lethal strains were recessive for sectoring. The lack of *ADE*3 integrants and gene conversions may be due to the way in which we constructed our plasmids. To construct pBK101, we ligated the 3.7 kb *Bam*H1/*Nhe*1 fragment of pDK255

containing *ADE3* into pRS314. This reduces the sequences flanking the *ADE3* gene by approximately 1.7 kb which may have reduced the possibility of homologous recombination.

In order to show that the requirement for the plasmid is due to the presence of *CHS3* and not to other sequences on the plasmid, putative UV mutagenized positives were transformed with pRS316, a *URA3* marked plasmid having a backbone identical to that of pBK102. Transformation with pRS316 should not effect the Sect- phenotype of true synthetic lethals because it does not carry *CHS3*.

In addition, the UV mutagenized putative positives were transformed with the *CEN* plasmid pCSD2-3, which carries a wild-type copy of *CHS3* but not *ADE3*. True synthetic lethals should sector when they acquire pCSD2-3 because either plasmid can satisfy the requirement for *CHS3*.

Of the 23 UV mutagenized putative synthetic lethal mutant strains tested, 10 behaved as defined for true synthetic lethals when transformed with the tester plasmids. Of these 10, four mutants grew very slowly (1b-1, 1d-1, 5c-2, 7e-1) and one mutant, (9c-5) failed to grow in top agar. For the present study, the remaining five mutant strains were further characterized.

The transposon mutagenized strains were mated with PRY483 sporulated, and tetrads analyzed for "missing" spore classes. Of the three

strains tested (DB2, DB3, DB4/DB6), the double mutant could not be recovered from DB4/DB6 (table 3). Recovered spores were tested for their ability to grow on Calcofluor White. Spores from the DB6 x PRY487 cross were also tested for histidine and leucine auxotrophy.

2. Agar Diffusion Assay for Drug Sensitivity

The 5 synthetic lethal mutant strains that were recessive and sectored only when transformed with a plasmid carrying *CHS3* and the 1 transposon mutagenized strain were tested for growth in the presence of cell wall specific drugs. The drugs included Nikkomycin Z, an inhibitor of chitin synthase III, Amphotericin B, a polyene which damages cell membranes (Odds, 1988), Tunicamycin, a specific inhibitor of N-glycosylation, Sodium Orthovanadate (vanadate), resistance to which often indicates defects in glycosylation (Kanik-Ennulat *et al.*), Hygromycin B, an aminoglycoside, Echinocandin and Papulacandin B, an inhibitor of β-1,3-glucan synthesis, Congo Red and Calcofluor White, dyes which bind to chitin and glucan fibrils, K1 Killer Toxin, which binds to β-1,6-glucan, and FK 506, a calcineurin inhibitor. Mutations in *FKS1* are known to be hypersensitive to FK506. The results are summarized in Table 2.

Relative to their respective parental strains (PRY485 and AWM3CΔ630) three mutant strains (3d-2, 13a-1, 13d-3,) showed increased sensitivity to Nikkomycin Z. Two mutant strains (12a-1, 12e-1)

showed increased sensitivity to Amphotericin B. Only 12e-1 showed increased sensitivity to Tunicamycin. One mutant strain (DB4/DB6) showed increased sensitivity to the β-1,3-glucan synthase inhibitors Echinocandin and Papulacandin B. All six synthetic lethal strains (3d-2, 12a-1, 12e-1, 13a-1, 13d-3, and DB4/DB6) showed increased sensitivity to the aminoglycoside Hygromycin B. Dean (1995) demonstrated that abnormal glycosylation results in sensitivity to aminoglycosides. This suggests that all five mutant strains are defective in glycosylation. Four of the synthetic lethal strains showed resistance to vanadate, (3d-2, 12a-1, 13a-1, and 13d-3). Only 13d-3 showed an increased sensitivity to FK 506. DB4, to K1 Killer Toxin sensitivity. DB3 was caffeine hypersensitive.

Four mutant strains (3d-2, 12a-1, 13a-1, 13d-3) showed resistance to Vanadate and sensitivity to Hygromycin B. This is characteristic of mutants defective in Golgi-specific glycosylation (Ballou *et al.*, 1991). According to Dean (1995) even mutants with defects in the early steps of glycosylation, are sensitive to Hygromycin B. Only the 12e-1 mutant strain showed hypersensitivity to Tunicamycin. This is also the only mutant that is not resistant to vanadate.

3. Isolation of Complementing Clone and Database Search

The Nikkomycin Z hypersensitivity of 13a-1 and 13d-3 was used to facilitate the cloning of the complementing insert from the *URA3* marked *CEN4* library. Initial sequence data obtained from the complementing insert was used to search existing databases. The gene mutated in 13d-3 was identified as *FKS1* which is identical to *ETG1* (Douglas *et al.*, 1994a), *CWH53* (Ram et al., 1994), *PBR1* (Castro *et al*, 1995), and *CND1* (Garrett-Engele *et al.*, 1995). The *FKS1* gene encodes a sub-unit of the *S. cerevisiae* β-1,3-glucan synthase.

For 13a-1, a BLAST query identified a sequence present on chromosome V. Since the library inserts average 9 kilobases in length, most contain several genes. The entire DNA sequence of this clone was downloaded and analyzed for open reading frames (ORFs). Using sub-cloned portions of the insert followed by complementation analysis, the mutated gene in 13a-1 was determined to be *ANP1*. The *ANP1* gene was initially identified in a mutant screen for resistance to aminonitrophenyl propanediol, (McKnight *et al.*, 1981). Subsequently, *ANP1* has been shown to be involved in Golgi retention of the "medial" enzyme Mnt1p (Chapman and Munro, 1994) and the late Golgi protease DPAP-A (Northwehr et al., 1996). *ANP1* is homologous to *MNN9* and *VAN1* (Kanik-Ennulat and Neff, 1990).

Unlike 13a-1 and 13d-3, 12a-1 showed no sensitivity to Nikkomycin Z The sensitivity of 12a-1 to Calcofluor white and Congo red, in addition to the acquisition of the ability to grow on SD^+ without uracil was used to select transformants with a complementing insert. A BLAST query identified the complementing clone (located on chromosome XIV). Sub-cloning and complementation analysis resulted in the identification of the complementing gene in 12a-1 as *SRV2* (*CAP1*, *END14*) which has been implicated in the transmission of cyclic AMP (cAMP)-mediated signals via the RAS/adenylyl cyclase pathway (Fedor-Chaiken et al,, 1990; Gerst et al., 1991; Wang et al., 1993) and more recently shown to bind to the SH3 domain of the actin binding protein, Abp1p. The *SRV2* gene may provide a link between growth signals and the cytoskeleton, (Drubin and Lila, 1997).

4. Complementation Analysis.

Plasmids carrying the isolated complementing gene were used to verify the mutant phenotypes as well as additional members of the complementation group. The plasmid p13d-3, containing the wild-type clone of *FKS1*, was used to transform all five synthetic lethal mutant strains, including 13d-3. The plasmid p13d-3 complemented the sectoring phenotype and drug profile of the original mutant, 13d-3, as well as two additional mutants, 3d-2 and 12e-1.

When p12a-1 or p13a-1 were used to transform the putative synthetic lethal strains, only the original mutant strain was complemented

for both the sectoring phenotype and drug profile. Thus, we have identified at least 3 different complementation groups as being essential in combination with a *CHS*3 deletion; *FKS1* (3 members), *ANP1* (1 member) and *SRV2* (1 member).

5. Cell Wall Composition

The cell wall composition of the mutant strains might suggest the cause of the synthetic lethal interaction with *chs3*. Strains were grown in YPD supplemented with radioactive glucose. Cells were harvested and fractionated (see Materials and Methods) and the radioactivity incorporated into the alkali insoluble fraction and the mannan cell wall fraction was determined.

The incorporation of label into the alkali insoluble fraction, containing alkali insoluble glucan and chitin, was 20-30% lower for the *fks1* mutant strains than for the control strains PRY485 and PRY487. The *fks1* strains showed a level of incorporation of label into mannan comparable to the control strains.

Incorporation of label into the alkali insoluble fraction for the *anp1* strain was approximately equal to the control strains but incorporation of label into mannan was reduced by 75%.

The cell wall composition data for the *srv2* strain was comparable to the control strains.

DB4 has increased mannose/glucose ratios relative to the parent strain AWM3CΔ630. The levels of N-acetylglucosamine are increased in DB4.

6. β-1,3-Glucan Synthase Activity

Glucan is a major component of the yeast cell wall. To better understand the synthetic relationship between our mutants and *chs3*, we measured their β-1,3-glucan synthase activity.

Compared to the control strain PRY485, all three members of our *fks1* complementation group, (3d-2, 12e-1 and 13d-3), show a significant reduction in glucan synthase activity; the activity being 15-38% of the control. This is consistent with previously published data (Castro *et al.*). The *anp1* mutant strain (13a-1) shows a less marked decrease in enzyme activity (66% of the control). Unlike the *fks1* and *anp1* strains, the *srv2* mutant strain (12a-1) shows an increase in enzyme activity; 126% of the control strain.

7. Other Synthetic Lethal Interactions

The synthetic lethality of the *fks1* complementation group along with the Nikkomycin Z hypersensitivity for two of the three mutants suggests that a simultaneous decrease in glucan and chitin is lethal. If true, one would expect *fks1* strains to be lethal in combination with other mutations that result in a loss of chitin synthesized by CHSIII. To test this, strain

PRY581 carrying the original mutant *fks1* from 13d-3 (but not the *chs3::LEU2* disruption) was mated to the *chs4-4::LEU2* strain PRY582, the *chs3::LEU2* strain PRY502, and the *csd3::TRP1* strain PRY404. Diploids were dissected and the tetrads analyzed for segregation of markers and viability (see table 4).

For PRY581/PRY582, 12 double mutant (*chs4::LEU2 fksl*) spores were expected. Based on the lack of Leu^+ Nikkomycin Z hypersensitive spores, no double mutants were recovered. Therefore, *fks1* is synthetically lethal in combination with a deletion of *CHS4*.

The cross of PRY581 (*fksl*) to PRY502 (*chs3::LEU2*) is a reconstruction of the original synthetic lethal strain. For PRY581/PRY502, 12 double mutant spores (*chs3::LEU2 fks1*) were expected but none were recovered. This result confirms the synthetic lethality of the double mutant, *chs3 fks1*. The synthetic lethality of *fks1* with both *chs3* and *csd4* supports the idea that a simultaneous reduction in β-(1,3)-glucan and chitin (specifically chitin synthesized by CHS3) is lethal.

For PRY581/PRY404, of the 8 double mutant (*csd3::TRP1 fks1*) spores expected, 5 were viable. Unlike *chs3* and *csd4* strains, *CSD3* deletion strains do have chitin synthase activity *in vitro*. The function of *CSD3* is not known. Perhaps an additional genes(s) is segregating in this cross which can substitute in some way for *csd3::TRP1*. Calcofluor sensitivity

should segregate 2:2 in this cross. Three tetrads were tested for their ability to grow on YPD plus calcofluor. Of the 12 spores tested, 10 were calcofluor sensitive. The excess of calcofluor sensitive spores is consistent with the idea that CHS3 is functional. Further experimentation is required to understand the interaction of *fks1* and *csd3*.

Our mutant screen has shown that *chs*3::*LEU*2 is synthetically lethal with *anp1*. The *ANP1* gene is homologous to *VAN1* and *MNN9*. Together, the three constitute a family of genes required for proper Golgi function in S. cerevisiae (Chapman and Munro, 1994). To determine whether or not the other members of this gene family have the same synthetic lethal interaction with *chs*3, heterozygous diploids were constructed with *mnn9* and with *van1*. Following tetrad dissection, we examined the segregation of markers and the viability of the spores. The results, summarized in Table 5 show that *chs3* is indeed lethal in combination with both *mnn9* and with *van1*.

J. Discussion

In order to identify additional genes involved in the regulation of chitin synthesis in *Saccharomyces cerevisiae*, we carried out two synthetic lethal screens to look for genes that when mutant, require a functional Chs3p for viability. Our first genetic screen identified UV mutations in three different complementation groups that cause yeast cells to require *CHS3* for viability. The second screen identified one transposon

mutagenized yeast strain, DB3, with a mutation in *ECM19,* to be synthetically lethal in combination with *CHS3.* Three of the UV synthetic lethal mutants isolated fall into a complementation group determined to be *FKS1* by library complementation and sequence analysis. *FKS1* encodes a subunit of β-1,3-glucan synthase which synthesizes 1,3-β-glucan, a major structural component of the cell wall. Mutations in this gene have been isolated in other screens designed to identify cell wall alterations e.g. hypersensitivity to FK506 (Eng *et al.*, 1994), resistance to Echinocandins (DeMora *et al.*, 1991; Douglas *et al.*, 1994b), and sensitivity to Calcofluor White (Ram *et al.*, 1994). As expected, all three of our *fks1* mutants have reduced β-1,3-glucan synthase activity relative to the control strain PRY485. The residual enzyme activity is thought to be due to *FKS2*, a homologue of *FKS1* (Fks2p is 88% identical to Fks1p).

A second complementation group, represented by one member, 13a-1, was determined to be *ANP1*. The *ANP1* gene encodes a protein that is part of a family of yeast type II integral membrane proteins that includes Van1p and Mnn9p (Chapman and Munro, 1994). Anp1p, Van1p and Mnn9p are required for proper Golgi function in *S. cerevisiae* (Jungmann and Munro, 1998). We found that *chs3* is lethal in combination with not only *anp1*, but also with *van1* and with *mnn9*. Jungmann and Munro (1998) have shown that Anp1p, Van1p and Mnn9p co-localize

within the *cis* Golgi and form two distinct complexes, each complex containing Mnn9p and either Anp1p or Van1p. Both complexes have α-1,6-mannosyltransferase activity. Yeast with disrupted Golgi function result in abnormal glycosylation (Chapman and Munro, 1994). Glycosylation mutants, such as *mnn* (mannan defective), *vrg* (vanadate resistant genes) and *alg* (asparagine linked glycosylation) have been shown to be resistant to Sodium Orthovanadate, and sensitive to Hygromycin B (Chapman and Munro, 1994). As expected, 13a-1 is resistant to Vanadate and sensitive to Hygromycin B. It has been shown that the *mnn9* family of proteins have reduced mannan. Consistent with this, cell wall composition analysis for 13a-1 shows 70% less incorporation of label into mannan than for the control strain, while incorporation of label into the alkali-insoluble cell wall fraction is about equal to the control strains.

Determination of β-1,3-glucan synthase activity in the 13a-1 strain showed a reduced level of enzyme activity relative to the control. For this strain, there is a concomitant glycosylation defect along with a decrease in enzyme activity.

Recently, Mondesert *et al.* (1998) reported the isolation of morphogenesis checkpoint dependent (*mcd*) mutants that are defective in growth but have normal actin organization. One of the genes mutated in the *mcd2* strains was cloned and identified as *ANP1*. *ANP1* mutant strains

are vanadate resistant, have defects in establishing growth polarity and are defective in glycosylation. Mondesert *et al.* suggest that an increase in N-linked glycosylation is needed not only for the mannoproteins required for cell wall construction during bud emergence but also to direct secretion to the presumptive site of bud emergence and to the emerging bud. Consequently the lethality of *chs3* and *anp1* may be due to a weakened cell wall which results from the decrease in mannan in the *anp1* strain or it might be due to a need for *N*-glycosylation of a protein/proteins as part of a signaling pathway involved in polarization of secretion during the cell cycle. Further study is needed to determine the exact nature of the synthetic lethality of *chs3* and the *mnn9* family of proteins.

A third complementation group defined by 12a-1, was determined to be *SRV2*. Srv2p is an adenylate cyclase-associated protein that may provide a link between growth signals and the cytoskeleton. *SRV2* encodes a 526 amino acid protein that has at least three functional domains. The N-terminal domain (aa 1-192) binds to adenylyl cyclase and is necessary and sufficient for the phenotypes associated with activated RAS. *SRV2* is required for RAS-activated adenylate cyclase activity (Fedor-Chaiken *et al.*, 1990) but mutations that make cell viability independent of the production of cAMP do not suppress the lethality of null alleles. Therefore *SRV2* must provide an essential function to the cell that is independent of the production of cAMP.

The C-terminal domain is required for normal cellular morphology and response to nutrient extremes (Gerst et al., 1991). The C-terminal domain binds to monomeric actin and has a cytoskeletal regulatory function *in vivo*, (Freeman *et al.*, 1996). The middle third of *SRV2* contains a proline rich region (aa 273-286) which has been shown to bind to the Src homology 3 (SH3) domain of the actin binding protein, Abp1p, (Lila and Drubin, 1997). *SRV2* may play a role in maintaining the integrity of cellular membranes. This would be consistent with its abundance and its localization to cell membranes (Field *et al.*, 1990) and cortical actin patches, (Lila and Drubin, 1997) which are thought to be regions of the cell that are actively growing.

The final mutant, *ECM19*, encodes a 112 a.a. protein of unknown function which contains no transmembrane domains and two stress protein regulatory elements. While little is known about this small protein, it is reasonable to speculate that it might have some role in up or down regulating Chs3p during environmental stress.

To identify genes directly associated with *CHS3*, the primary chitin synthase, we conducted synthetic lethal screens with UV mutagenized cells and the "droopy bud" mutants. We have identified 6 mutant strains, representing 4 complementation groups, which have mutated genes which are lethal when in combination with *CHS3*. However, these screens are not saturated. The remainder of the transposon mutagenized strains need to

be tested for synthetic lethality. There are at least 5 other mutants identified in the UV screen which have yet to be characterized. In addition, many more than expected genes have yet to be isolated. As a result, further study is needed to be done to identify additional genes which interact genetically with *CHS3*.

K. Tables

Table 1

Yeast Strains Used

Strain	Genotype
PRY398	*MAT*a *ade2 ade3 his3 leu2 lys2-801 trp1*
PRY436	*MAT*a *ade2 ura3 leu2 mnn9::URA3 suc2-9 pep::CAT gal2*
PRY449	*MAT*a *his4-539 ura3-52 lys2-801 van1-18*
PRY485	*MAT*a *ade2 ade3 his3 leu2 lys2-801 trp1 chs3::LEU2*
PRY487	*MAT*a *ade2 ade3 his3 leu2 lys2-801 trp1 chs3::LEU2* + *pBK102 (CEN6 ADE3 TRP1 CHS3)*
PRY502	*MAT*a *ade2 ade3 ura3 leu2 trp1 csd::LEU2*
PRY512	*MAT*a *ade2-101 his3-D200 ura3-52 leu2-D1 lys2-801 trp1d1 chs3::LEU2*
105.1A	*MAT*a *ade2 ade3 ura3 leu2 CHS3 trp1 fks1*
72.2A	*MAT*a *his3-D200 trp1-D1 ura3-52 leu2-3 112 csd4-4::LEU2*
DB2	*MAT*a *ura3-639 leu2-3 2-112 his3-11 3-15 ecm5::Tn3::LEU2::lacZ* cir^0
DB3	*MAT*a *ura3-639 leu2-3 2-112 his3-11 3-15 ecm19::Tn3::LEU2::lacZ* cir^0
DB4	*MAT*a *ura3-639 leu2-3 2-112 his3-11 3-15 ecm20::Tn3::LEU2::lacZ* cir^0
DB6	*MAT*a *ura3-639 leu2-3 2-112 his3-11 3-15 ecm19::HIS3* cir^0

Table 2

Drug Profiles of *CHS3* Synthetic Lethals

Strain	Nikko	Amph	Tuni	Echino	Paplu	Hyg	Van	FK506	K1	CFW/CGR
3d-2	HS	S	-	-	S	S	R	-	S?	HS
13d-3	HS	-	-	-	S	S	R	S	-	HS
12e-1	-	HS	HS	ND	S	HS	-	-	S	S
12a-1	-	HS	-	-	-	S	R	-	-	HS
13a-1	HS	-	-	-	-	S	R	-	S	S
DB4/DB6	-	ND	ND	HS	HS	HS	ND	ND	HS	HS

Drug sensitivities have been determined by measuring zones of inhibition relative to parent strain.

Nikko - Nikkomycin
Amph - Amphotercin
Tuni - Tunicmycin
Echino - Echinocandin
Papalu - Paplucandin
Van - Vanadate
Hyg - Hygromycin
K1 - K1 Killer Toxin
Van -Vanadate
FK506
CFW/CGR - Calcofluor White/Congo Red

- = same as wt
S= sensitive
HS= hyper sensitive
ND = not done
R =resistant

Table 3

DB4/DB6 X PRY487

Cross	#Expected/ #Viable	%Viable	#*chs ecm19* Expected	Actual	*% chs ecm19 Lethality*
ecm19::Tn3 X chs3::LEU2[1]	38/60	63%	unclear	unclear	unclear
ecm19::HIS3 X chs3::LEU2[2]	58/80	73%	22	0	100%
5PD:7:NPD:8TT					

[1]All recovered spores from this cross were struck for singles on Calcofluor White plates and examined microscopically determine whether or not they were *chs3* mutants.

Table 4

Other Synthetic Lethal Interactions

Cross	#Expected	#Actual	%Lethality
fks1xchs3::LEU2 *1PD:NPD:8TT*	12	0	100%
fks1xchs4::LEU2 *2PD:3NPD:6TT*	12	0	100%
fks1xchd3::TRP1 *3PD:1NPD:5TT*	8	5	37%
	Hieter Background		
fks1 x chs3::LEU2 3PD:0NPD:5TT	5	0	100%
fks1 x csd4::LEU2 3PD:2NPD:2TT	6	0	100%
fks1xcsd4::LEU2 3PD:1NPD:4TT	6	0	100%

Table 5

Synthetic Lethality

13d-3 X PRY398

Genotype	#Viable/#Expected	%Viability
chs3::LEU2 mnn9::URA3	0/11	0%
chs3::LEU2 van1-18	0/9	0%

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V. Conclusions

We embarked upon two screens in an attempt to identify genes involved in cell surface assembly, specifically genes involved in the synthesis of chitin. In the first screen we decided to cast a wide net in an effort to isolate a variety of genes in the hopes that some would be obviously related to the function or regulation of the chitin synthases. The second screen was more focused, looking somewhat more directly at interactions with *CHSIII*.

It is clear from these studies that there is still much more work to do in the search for genes involved in cell surface assembly. In the first screen, we suprisingly did not isolate any chitin synthase mutants but we did identify glucan synthase genes. The absence of these classes, I feel, speaks to the lack of screen saturation rather than a poor screen construction. Mutations in chitin synthase result in cells which are highly resistant to CFW and CGR.

That said, there are several ways in which the CFW/CGR screen could be improved.

First, in addition to the large haploid screen, a diploid screen needs to be initiated to identify lethal transposon insertions.

Second, it is unclear whether or not the initial water dilutions of the cells was detrimental to the screen. We did identify osmotically sensitive

mutants; however, we probably killed cells that were severely sensitive. The initial dilution conditions could easily be 10% sorbitol rather than water. Third, the transposon mutagenesis itself has problem. It is known that many cell surface assembly genes are members of large redundant families where a phenotype is only seen when there are multiple mutation events. These genes would be missed by a screen such as ours. A potential solution would be to begin with strains containing a known mutation, and transposon mutagenize them.

Fourth, it is known that the Tn3 library contains several integration "hotspots" (Burns, personal communication). We screened some 15,000 mutants in an effort to cover the genome and deal with the "hotspot" problem. However, it is possible that we have miscalculated and several thousand more mutants need to be screened to account for the "hotspot" loss.

Finally, one of the dangers in conducting a non-specific screen is that many mutations are identified which are indirectly associated with the system studied. Since our screen relied on the uptake of Calcofluor White and Congo Red, transport mutants might be included. This fundamental criticism of non-specific screens point to fact that an additional initial screening mechanism was needed to eliminate the noise. We addressed some of those concerns by screening against petites. However, given the

number of "global regulatory" genes identified, additional precautions needed to have been taken.

The screen is far from saturated and needs to be repeated. Aside from that, there are several interesting genes that have been identified which need to be studied further. The "droopy bud" mutants need to be further characterized. There is much more work to do before cell surface assembly in *Saccharomyces cerevisiae* is completely understood.